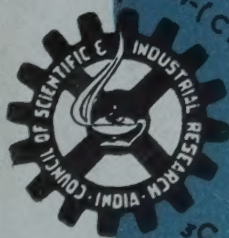


# A

# ntibiotics

## A Symposium

Council of Scientific &  
Industrial Research





The discovery of antibiotics has been hailed as one of the greatest milestones in man's fight against disease. Several hundred antibiotics have been isolated, but only about twenty of them have found application as chemotherapeutic agents. New sources of antibiotics are being explored and new applications are being discovered, e.g., treatment of animal and plant diseases, animal feeds, and preservation of biological materials and foods. Active centres of antibiotics research exist in almost all countries. In India, particularly since the establishment of commercial production at Pimpri, the tempo of research on all aspects of antibiotics has increased. A survey of the progress made and the results achieved will be found in this collection of papers presented at a symposium on the production, utilization and mode of action of antibiotics, held at the Hindustan Antibiotics (Private) Limited, Pimpri.

The papers have been classified under the following groups: (i) screening of soils and plants for antibiotics; (ii) production : raw materials and methods of manufacture; (iii) biological assay of antibiotics; (iv) byproducts; (v) chemistry and biosynthesis; and (vi) applications. This collection of papers should prove useful to all those interested in antibiotics : the research worker, the manufacturer, and the specialist interested in clinical applications.





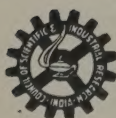




# ANTIBIOTICS

*Their Production, Utilization and  
Mode of Action*

A SYMPOSIUM HELD AT  
THE HINDUSTAN ANTIBIOTICS (PRIVATE) LTD., PIMPRI  
MARCH 27-30, 1956



Council of Scientific & Industrial Research  
New Delhi  
1958

# ANTIBIOTICS

For the purpose of the Council of Scientific & Industrial Research

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## PREFACE

The Biochemical Research Committee recommended at its midyear meeting held on August 22, 1955 at Mysore that a symposium on *Antibiotics—Their Production, Utilization and Mode of Action* be held at Pimpri, under its auspices, in collaboration with the Pharmaceuticals and Drugs Research Committee. The Governing Body of the Council of Scientific & Industrial Research gave its approval to this in September 1955.

The Biochemical Research Committee, at its Sixteenth Annual Meeting in Lucknow on December 21, 1955, appointed a steering committee consisting of Dr. K. Ganapathi and Dr. D. L. Shrivastava with Dr. A. Sreenivasan as convener for working out the details and arranging the symposium. Various institutions and workers in the field of antibiotics were approached through circular letters, personal communications and press notes to contribute papers for the symposium. The response was very encouraging and over 60 papers were received. The Biochemical Research Committee is deeply appreciative of this co-operation and would wish to express its thanks to all those who have contributed papers to the symposium as well as the organizations and institutions for sending delegates to the symposium.

The Committee is very much indebted to Col. J. R. Dogra, Managing Director, and to Dr. K. Ganapathi, Superintendent Research, *Hindustan Antibiotics (Private) Ltd.*, Pimpri, for much valuable help and for acting generally as hosts to the participants and invitees to the symposium.

Central Food Technological  
Research Institute, Mysore  
April 10, 1956

V. SUBRAHMANYAN  
Chairman,  
Biochemical Research Committee





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## Welcome Address

COL. J. R. DOGRA

Managing Director, Hindustan Antibiotics (Private) Ltd., Pimpri

I have a very short, but extremely pleasant, duty to perform this morning, that is, to welcome you to Pimpri.

When the Council of Scientific & Industrial Research desired to have a meeting of the Biochemical Research Committee and the Pharmaceuticals & Drugs Research Committee jointly to discuss and study *Antibiotics—Their Production, Utilization and Mode of Action*, I was only too happy to grab the opportunity and to invite them to Pimpri. I considered it not only an opportunity but a very appropriate venue for their activities. This undertaking, as you will see, is the first antibiotics factory started under the joint auspices of the Government of India and the United Nations Organizations and a fairly good sized Antibiotics Research Centre. It is significant that the management recognizes the importance of research in an industry of this nature and has, therefore, withheld no financial assistance to enable us to equip and man the Research Centre. Research problems have been posed and work started. Considerable success has been achieved in some directions. Some of their achievements will be presented at the symposium.

We are not quite settled as yet. We are, therefore, only able to offer hospitality and facilities for your activities in a rather unsatisfactory manner, but as a scientist I know that scientists do not lay emphasis on creature comforts. They desire opportunities for meeting other scientists engaged in similar pursuits in order to exchange ideas, observe the scientific work conducted and thus contribute to the advancement of science.

I do hope that the distinguished scientists who have gathered together will help our team of scientific workers to solve their immediate and distant problems—problems which arise from manufacturing processes and other matters—and in turn will take back with them our plans for scientific research to enable them to experiment further.

On behalf of all the workers of this undertaking, I offer you all our warmest welcome.

# Inaugural Speech

SHRI K. C. REDDY

Union Minister for Production

I feel happy to be here today to inaugurate this symposium on *Antibiotics—Their Production, Utilization and Mode of Action* organized by the Biochemical Research and the Pharmaceuticals & Drugs Research Committees of the Council of Scientific & Industrial Research (CSIR).

The CSIR has established in the country, during the last few years after our Independence, quite a number of research laboratories—14 of them—under the inspiring guidance of our Prime Minister whose faith in science and technology is well known. We can progress to prosperity only through science and technology. Of course, we should utilize the result of science for moral and peaceful purposes only. If we misuse them we will be imperiling humanity and civilization. The world today is, as you know, in the suffocating grip of the fear of nuclear weapons of war. But what a different world it will be if we can use, in an expanding degree, atomic power for peaceful purposes.

## Discovery of Antibiotics

The discovery and development of antibiotics has been hailed as one of the greatest contributions to science and technology during the last two decades. Antibiotics, which are chemical substances produced by micro-organisms and which when used in very minute quantities cause the inhibition of growth of micro-organisms, are now an indispensable weapon in the medical armoury. These are in fact a boon to humanity. From the practical point of view many of the most dreadful diseases with very high mortalities have now been brought under control. The surgeon has been relieved of one of his biggest headaches—the onset of fatal sepsis following surgical operations. Briefly, as a result of the increasing knowledge and use of antibiotics the average life of people of even under-developed and backward countries has increased and the population graph is showing an upward trend. These substantial results have been obtained, it must be remembered, in a matter of only about 15 years, even when the present knowledge of the potentialities of these drugs are known only to a limited extent.

Experiments and research have proved that antibiotics can be used in the treatment of cattle diseases and plant pests as well. I understand that many



of the diseases of cattle are amenable to treatment with these antibiotics. To give but one example, the use of penicillin for treatment of milch cows suffering from infectious mastitis results both in the alleviation of suffering and an increase in milk supply. I am also told that penicillin when administered in small quantities stimulates the growth of young animals. This field is, therefore, of immense potential value to us in India with probably the largest number of under-nourished cattle.

I understand that these drugs could also with advantage be used in agricultural pest control. Naturally, we in an agricultural country are immensely interested in this field of work ; for, if successful, this could revolutionize our economy which is mainly agricultural.

The potentialities of antibiotics have not yet been fully realized and thousands of scientists and workers are busy all over the world examining moulds and micro-organisms as potential sources of antibiotics. We have here in Pimpri a Research Centre attached to the penicillin factory and our scientists like others are striving hard. We will do well to realize that no discovery however wonderful could be useful unless its benefits are made available to the common man at as cheap a cost as possible. The efforts of scientists, therefore, must be not only to discover useful drugs, but also to help make them cheap and abundant. This calls for close collaboration between the scientists, and the technologists and production units.

### Penicillin

The most important among the antibiotics is still undoubtedly penicillin. To make this important and useful drug available to the common citizen of our country, the Government of India with the active help and guidance of WHO and UNICEF, set up this manufacturing unit some time ago. It is probably the best penicillin manufacturing plant east of Suez. During the short period of its existence, the factory, I am glad to say, has made commendable progress. And I am thankful to the scientists and workers associated with the *Hindustan Antibiotics* for their sustained efforts to manufacture penicillin in larger quantities and at cheaper cost. I congratulate them on their efforts. I would like to take this opportunity to thank the Bombay Government for their assistance in bringing this factory into existence. Having entered the field later, however, we have to compete with formidable rivals with years of experience and a fund of scientific knowledge. I hope that with the efforts and ingenuity of our scientists and workers, we shall be able to hold the ground. I consider this factory the starting point in our efforts to build up a large antibiotics industry in our country. For, with the experience gained here we could build other factories for manufacturing useful drugs to serve the common man.

### Other Antibiotics

Another antibiotic in which we are greatly interested at present is streptomycin for the treatment of tuberculosis. I am glad to say that we have decided to take up the production of this drug in this factory and we hope to

have a streptomycin plant here within about two years from now. There are a number of other antibiotics which we would like to take up as early as possible. The manufacture of these, however, are the exclusive monopoly of some private firms outside the country. Probably because of this monopolistic control these antibiotics are priced much higher than penicillin and streptomycin, and this price factor naturally restricts their use. If the common man is to have the benefit of useful drugs such monopolistic controls and patents will have to go.

### Symposium

I hope that good results will follow the deliberations of the symposium which I have the privilege to inaugurate today. In this assembly we have scientists, technologists, producers and consumers. I am sure their deliberations will prove of advantage to the manufacturing team and to others also. Those coming from various laboratories and plants in the country have now an opportunity of getting personally acquainted with the work here, appreciating the problems confronting the workers and offering their own suggestions and ideas. Manufacturing teams also have the opportunity of placing their problems before the eminent scientists and getting their reaction. This unit is State-owned and, in a special manner, belongs to all who have assembled here and it is in the interests of you all to see that this factory really develops into a most useful institution in the country and serves as a forerunner to yet more factories.

Finally, let me express my hope that symposia of this kind will not be occasional gatherings but will form a regular annual feature. I have now great pleasure to inaugurate the symposium and wish you success in your deliberations.

## *Presidential Address*

DR. JIVRAJ N. MEHTA

Minister for Finance, Prohibition & Industries, Bombay State

I am indeed very grateful to be asked to preside over the first symposium on *Antibiotics* to be held in the premises of the first antibiotics factory built in India. It may interest you to know that it became my privilege to give some thought to the scheme for the production of penicillin in the first instance when the Council of Scientific & Industrial Research (CSIR) had appointed in 1946 a Sub-Committee consisting of myself, Col. (now Major-General) Sokhey and Prof. Subrahmanyam of Bangalore (now of Mysore) to report on the possibilities of sponsoring such a project in India. The question was discussed at great



length then which resulted in the Planning & Development Department of the Government of India to decide and send a deputation to visit U.K., U.S.A. and Canada to make an exploratory investigation into all aspects connected with penicillin, sulpha drugs and anti-malarial drugs. The confidential report of this deputation submitted in January 1947 by Sokhey and Ganapathi, for reasons that you can very well understand—we were not free in those days—did not receive appropriate consideration and for all practical purposes it was completely forgotten. Later on, when I took charge of the Medical and Public Health Department of the Government of India in August 1947, one of the things that received high priority from me was the question of penicillin, sulpha drugs and antimalarial drugs in spite of the fact that most of my time had to be devoted to overcoming difficulties arising out of the unprecedented refugee problem. While preparing plans for the implementation of the essential drug schemes, a proposal was submitted to secure a penicillin plant from America for the ridiculously small sum of about \$40,000 of which only \$8,000 were to be paid immediately and the rest of \$32,000 to be spread over ten annual instalments. The offer proposed to provide training for Indian personnel at no extra cost. This proposal was studied by Dr. J. C. Ghosh, the then Director-General of Industries and Supplies, who suggested serious consideration of this in preference to the Rs. 192 lakhs scheme proposal of Col. Sokhey and Dr. Ganapathi. Col. Sokhey's views were obtained on this and later there were several conferences between the Ministry of Health and the Ministry of Industry & Supply almost immediately afterwards. I accepted the advice tendered by Col. Sokhey not to consider the purchase of a cheap second-hand plant (the plant was not really very old being less than 2 to 3 years old) and obtained sanction for sending a second deputation to study the question once again led by Col. Sokhey with Dr. Sankaran and Dr. Ganapathi as members. The members of the deputation left in March 1948 and returned in August the same year. Their report was submitted in October 1948 and immediately afterwards it was decided to implement the contents of the report. A Swedish delegation came over in January 1949 and in February a preliminary draft agreement was signed between the Swedish firm of *Karbolaget* and the Government of India. After that it was hoped that in less than two years we would have seen the completion of the project. As they say much water has flowed under the bridge since then. UNICEF offered financial and WHO technical aid which resulted in the breaking up of our ties with the Swedish firm of *Karbolaget* as well as with the Bombay Government who ceased to be a partner with the Government of India in the joint venture, as it did not think that the arrangements made through the UNICEF and WHO would lead to an early development of the penicillin factory instead of through *Karbolaget* and *Mercks*. Unfortunately the fears of the Bombay Government proved true and instead of the factory materializing in 1952, as was then hoped, it materialized in 1955, i.e., 3 years later, during which period we have lost over 6 crores of rupees of foreign exchange. However things progressed, though slowly, and we are indeed glad that the first commercial production started in March 1955 and full production in August of

the same year. Col. Dogra and his colleagues are to be congratulated for this achievement. Within six months of starting production, we are now having a symposium on the various aspects of penicillin, including its production, which had so far been kept as closely guarded secrets. For this symposium we have received well over 60 papers contributed at very short notice. I do hope more symposia will be organized which will help in disseminating what might be designated as the newer knowledge of therapeutics, just as vitamins heralded the newer knowledge of nutrition.

Though this newer knowledge of antibiotics beginning with penicillin is hardly a dozen years old, it has grown enormously and at a very rapid rate, incomparable with any other therapeutic product or for that matter with any other kind of consumer goods as can be seen from Table 1 which gives the annual production in U.S.A. and U.K.

Consequent on increased production, the price of the drug has progressively and rapidly dropped down from \$20 a mega unit to only 3½ cents, i.e., about only 4 annas a mega unit on the basis of wholesale price.

The common man has thus been enabled to obtain it with his limited income and the health authorities have been provided with a powerful drug to bring down morbidity and mortality rates which in the past cost governments as well as the public enormous sums of money.

After seeing the above figures one is tempted to compare the plans of production of penicillin in India with those in U.S.A. and U.K. America with a healthy population of only 150 million people, is producing 645 million mega

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TABLE 1—PRODUCTION OF PENICILLIN IN U.S.A. AND U.K.  
(million mega units)

YEAR	U.S.A.	U.K.	WHOLESALE PRICE PER MEGA UNIT
1943	0.021	Nil	20.00
1944	1.633	0.036	5.00
1945	7.125	Not known	2.00
1946	25.809	3.120	0.50
1947	30.640	4.888	0.50
1948	90.501	9.672	0.30
1949	138.100	18.616	0.20
1950	219.903	36.400	0.13
1951	324.293	61.204	0.15
1952	350.000	62.888	0.15
1953	530.865	94.343	0.10
1954	645.750	124.450	0.08
1955	..	..	0.05
1956	..	..	0.035

---



units a year and the United Kingdom of 40 million people, equally healthy, over 124 million mega units. I believe the present production capacity of the Pimpri plant is about 16.5 million mega units per annum. Considering the population of 360 millions in India and the adverse circumstances of climate and poverty both combining to increase morbidity and mortality, our requirements of penicillin should be over twice that of U.S.A. or say well over 1,000 million mega units. I believe there are plans on hand for having a 60 per cent expansion at Pimpri (about 25 million mega units) which is hoped to be accomplished within this year. Even then it could be seen that it is only a small fraction of our needs. The second Five-Year Plan may probably raise this by perhaps twice this quantum. But this in my view is barely sufficient. One of the officers of the Union Ministry of Health, after an on-the-spot survey all over India, reported in 1952 that our requirements may be as much as 140 million mega units. The report of the committee appointed to determine the types of penicillin to be produced at Pimpri and related matters concluded that the Government should plan for the production of 80 million mega units per year by the end of the second Five-Year Plan. There seems therefore a good case for greater production of penicillin. This raises the controversial question of private and public sectors as well as single or multiple units of production spread over the country. I am sure with the able guidance of our Planning Commission we will solve this problem to the satisfaction of all sections of the population.

I would, however, like to make a strong plea for what I call a policy of planned and integrated chemical and pharmaceutical industry. We should try and produce within our country all the essential raw materials as well as those required in large quantities. Such integrated units should be spread round the country for strategic reasons as well as for satisfying regional aspirations. In 1948, when I was in the Ministry of Health, I had made several specific proposals in this connection and I hope that the time has now come for their due consideration and early implementation. I am glad to know that India now produces all the corn-steep liquor required for the production of penicillin which was being imported before. It is also essential that the other raw materials, such as solvents and precursors, should also be produced in the country before long.

The next point I would like you to consider is the newer developments in penicillin therapy. When we started on our plans in 1947 we knew of only freeze-dried and later crystalline soluble penicillins which had to be painfully injected every four hours or so. Later, in 1948, we heard of procaine penicillin requiring only one injection a day. These are the two types now being made at Pimpri. But we have already on the market a newer preparation Benzathine penicillin or Bicillin, of which only one injection is needed in 3 weeks and sufficient to cure many diseases amenable to penicillin. It is certainly a great improvement in penicillin therapy in a country with a low ratio of doctors to population. I do hope Pimpri will be alert enough to produce this as early as possible. I believe also that there have been still more radical developments and oral therapy is now possible with what is

known as phenoxymethyl penicillin or penicillin V. This, if effective enough, is ideal for India. I hope that this too is being considered.

While I am here stressing the need for utilizing penicillin and its newer clinical varieties, I will be failing in my duty if I do not focus your attention to the possible dangers in its indiscriminate use. You are aware that some bacteria develop resistance to drugs and reports are often made that such resistance is on the increase. Research work should be undertaken to counteract this tendency. There is also no doubt that a considerable amount of penicillin is wasted unnecessarily. When penicillin is costly and produced in limited amount in the country, it should be our duty to counsel economic usage of this drug.

I will now consider other antibiotics. All are aware that India has a very large population suffering from tuberculosis. Streptomycin is a powerful drug for relief from this disease. Its production should therefore be undertaken as early as possible. I believe it is included in the second Five-Year Plan.

There are then some other newer antibiotics under the name of tetracycline, chlortetracycline, oxytetracycline, etc. These are also useful and have definite place in therapy. Whether their manufacture in India is to be taken up is a question which should be settled as early as possible.

Even with all these newer antibiotics, I understand we have only started scratching the surface of this rich field. Many more are in the offing and I do hope that the young scientists of Pimpri and elsewhere in the country will make a contribution to the well-being of the world by discovering more potent and less harmful antibiotics. The scientists at Pimpri have been provided with adequate facilities and they would be failing in their duty if they do not add substantially to their knowledge in this field of antibiotic therapy. They should not be content with the mere production of penicillin, howsoever much it may save our foreign exchange. We should as a matter of fact be able to enrich the world with newer knowledge gained in this organization. Research is certainly a gamble, but all the same, planners and administrators are not likely to be so generous with funds if results are not forthcoming in course of time.

Lastly, I want to touch upon another aspect that has arisen as a consequence of the production of penicillin. We have established here a new industry based on fermentation technique. By this technique we produce not only valuable antibiotics but also other by-products. For example, the mycelium is a good cattle and chicken feed as well as a fertilizer for plants. Production of streptomycin gives a by-product, vitamin B<sub>12</sub>, which is very very expensive. We can also extract from these mycelia ergosterol which on irradiation gives vitamin D. Then we have other fermentative processes producing vitamins; riboflavin and ascorbic acid are now based on fermentation techniques. I am pointing these out to show how knowledge is getting diversified into many fields. One should recognize these broadening horizons and specialists should be willing to learn and investigate newer fields, however remote they may be to what they may begin with.

I wish the workers at Pimpri all happiness in their work and success in life.



## Opening Address

MAJ.-GEN. S. S. SOKHEY

Chairman, Pharmaceuticals & Drugs Research Committee, CSIR

I am glad to be here today to take part in this symposium on *Antibiotics*. My pleasure goes beyond the treat we are going to have in listening to a large number of papers testifying to the good work being done in our country in the new science of antibiotics. To me *Hindustan Antibiotics (Private) Ltd.*, who is our host today, is more than our meeting place. It is a dream come true. And I am glad to see Dr. Ganapathi and Dr. Macpherson in the audience who, I am sure, share my feelings. This is no personal reverie that I am referring to. The scientific world as a whole and the development conditions of our country needed an open penicillin plant, free of commercial restraints, so that it could be used as a training centre for WHO scholars and other scholars nominated by their own Governments to enable such Governments as desired it to put up their own penicillin plants and establish in this field of science an open collaboration to the great advantage of science and technology. This is the first open penicillin plant outside the socialist countries and as such it is a unit of great international, scientific and technical importance.

It is true that it has taken over ten years to bring this idea to fruition. On the basis of the work done on a pilot plant scale at the Haffkine Institute to produce sulphonamides and anti-malarial drugs, it became clear that these drugs could be made at a fraction of the cost of imported drugs. And what is more, it gave us assurance that the country had the talent and the capacity to undertake such a task for the good of our people. Dr. Ganapathi and his colleagues had worked out large scale production methods of sulphathiazole, sulphadiazine and sulphamerazine and had taken out patents in the name of the Bombay Government. We did not believe in patents ; they were intended not to prevent our colleagues from making these drugs if they wanted to, but as a protection against foreign firms preventing us to make them. We had also done work on the production of penicillin by surface growth methods, and it was in 1943 that I put up a proposal to the Bombay Government and later in 1945 to the Central Government, that we should make an effort to make our country self-sufficient in essential drugs and make a start by putting up plants to manufacture penicillin, sulphonamides and anti-malaria drugs. These drugs represented the great advance made by

modern therapeutics, and between them they would take care of three-fourths of all the illness that occurs in the country.

The proposal was well received and the Planning & Development Department of the Government of India in 1946 deputed Dr. Ganapathi and myself to go abroad and prepare workable projects for the manufacture of these drugs. It was easy to tackle the sulphonamides and anti-malarial drugs part of our assignment, as we had been producing sulphonamides from raw materials on a pilot plant scale at the Haffkine Institute since 1943. All that was needed to be done was to contact firms in Western Europe and America making chemical plant equipment, choose the equipment best suited for our purpose and obtain quotations.

But the penicillin part of the project presented difficulties. We had worked only on surface growth methods of penicillin production, but knew little about submerged fermentation methods, which had just come into use and were the preserve of private enterprise, and all doors were closed to us. But here too luck came our way. Toronto University had a penicillin plant of its own under the charge of Dr. Macpherson. This was open to us as any scientific laboratory and we were welcomed along with scientists from some other countries on behalf of UNRRA. Dr. Macpherson took great interest in the idea of a penicillin plant for India and gave us every assistance. There we studied large scale methods of penicillin production and then visited three large plants in U.S.A. and prepared a project report giving the cost of putting up a plant and estimates of costs of production. What is more, we brought back with us a very generous offer from Dr. Smith, President of the Toronto University, that he would be agreeable to take 6 to 8 scientists and technicians deputed by the Government of India in the Toronto plant during 1947 for training, so that they could master the technique of production and design a penicillin plant. We were thus being enabled to put up a plant ourselves without having to pay high fees and royalties to commercial firms. But it was made clear to us that this privilege was open to us only during 1947.

It must be stated here for record that as a result of the meeting of a Committee of the Council of Scientific & Industrial Research in the Royal Institute of Science in Bombay in 1945, a British officer was deputed abroad and just before we submitted our report, he advised Government that it should not undertake penicillin production as it would be quite beyond its capacity.

However, as our Government was busy with other pressing things—and not because of the advice of British experts—this project was not implemented and the year of privilege offered by Toronto University expired. So in 1948 I approached the Government to permit us to go abroad again to bring the project up-to-date in view of the very striking advances in extraction which had taken place in the meantime and to make fresh contacts to find a training place for our workers. Dr. Jivraj Mehta who was Director-General of Health Services, took keen interest and deputed me, Dr. Ganapathi and Dr. Sankaran. We were again lucky and were offered generous assistance to train our scientists and to plan a plant by a Swedish firm—*Karnbolaget* of Stockholm—for a small



fee of Rs. 2 lakhs. Then we visited a few plants in U.S.A. We contacted firms making equipment and drew up a project giving complete list of equipment needed for the three drugs, costs and blue prints of tentative buildings and layout.

We showed that we could put up a complete complex of plants ourselves at a cost of about Rs. 2 crores, without having to hire the services of a foreign firm. We also showed that the cost of production of 100,000 lb. of sulphonamides, 100,000 lb. of anti-malarial drugs and 4.8 million mega units of penicillin per year would be Rs. 90 lakhs against Rs. 3 crores which the country was then spending on importing those drugs. We also showed from the actual quotations that if we used the services of an American firm to help us to put up the plants, it would cost us an additional 2 million dollars as fees for services, royalties, etc.

I am sorry to say that this effort also failed although our Prime Minister was taking a personal interest in the project and the Bombay Government was collaborating to give all assistance and to share the cost of the project. Government did take the preliminary steps and entered into an agreement with *Karbolaget* and sent two workers to the plant at Stockholm. Four more were to be sent, but *Karbolaget* informed us that they had entered into an arrangement with a big American firm and would not be able to collaborate as fully as we desired. They recommended to us that we also should join up with this American firm. Enquiries were made and we found that we would have to pay very large sums every year for 15 years as royalties to this firm and what was still worse, it would have to be a closed penicillin plant. A plant working under commercial secrecy could not be used for training purposes. We reacted adversely to this proposal.

At that time I left for Geneva as I was offered the post of Assistant Director-General, WHO, which I accepted. This gave me a fresh opportunity to help the project. I got the WHO interested in helping member nations to put up penicillin plants of their own. To raise funds for the purpose we contacted UNICEF, a much richer organization than WHO, and were glad to find the chairman of their Executive Board, Dr. Rajchman, keenly interested and willing to help. A joint meeting of WHO and UNICEF was arranged at which WHO and UNICEF jointly resolved to assist member nations such as desired it, to put up their own antibiotic plants; UNICEF would provide the equipment and WHO, the technical knowledge. To give effect to this scheme I was authorized to organize the Section of Antibiotics under my charge at Geneva. The main requirement for organizing the Section was to find technical experts. They were not easy to find as they were employees of private firms. These firms not only would not let us employ any of their men, but actually warned us against helping nations to put up their own plants. Luck was with us again and the Toronto University played a fairy god-mother and Dr. Defries, Head of the Public Health Department of the University, made available the services of Dr. Macpherson and allowed him to come to Geneva to take charge of the Section.

Then in 1950, we prepared a penicillin project for India on the lines worked

out in 1948. Towards this, UNICEF agreed to allot about 850,000 dollars and WHO, about 350,000 dollars. Our Prime Minister and the Health Minister showed great interest in the project and the Government of India accepted to collaborate with WHO and UNICEF in 1951 to establish this penicillin plant as an open plant which could be used for training WHO and other scholars and would collaborate with such other plants as WHO may organize elsewhere. During the last two years the responsibility of WHO was taken over by the Technical Assistance Administration of the United Nations (UNTAA).

You can see how beneficial this arrangement is, because India itself will need to establish three or four more similar plants in the country before very long, and other Asian countries will desire assistance. And what is more, we shall be able to collaborate with open penicillin plants for scientific research. As a result of this collaboration with UNTAA and UNICEF you have before you a very attractive and very efficient, absolutely modern plant in the *Hindustan Antibiotics (Private) Ltd.*, and what is more, operated entirely by Indians. And you will hear during the symposium what a great success our workers have made already in the short space of time they have been working the plant.

I fully share with Dr. Jivraj Mehta the regret about the delay that has occurred in putting up the plant because of the change in plans. I know he has taken keen interest in the scheme from the very beginning and done everything to help and the delay must be galling to him as to all of us. I do not think, however, even if we had linked up with the American firm, we could have erected the plant by the end of 1952. However, even a little delay is to be regretted, but it could not be avoided. If we had gone with the American firm, our plant would have been a 'closed' plant and we would have had to pay several crores in royalties.

I am personally, however, a little sad that the chemical part of the plant as originally conceived is missing. It would have manufactured besides sulphonamides and anti-malarials, some chemicals needed for penicillin production, and would have made the cost of production still lower. But even now we can take pride in the work our men are doing in that they are producing penicillin at a fully competitive price.

You will agree with me that the *Hindustan Antibiotics (Private) Ltd.* is a genuine step in the direction of self-sufficiency of our country in essential drugs, and the plant is providing our scientists and technicians a chance to show what Indian talent can do. We must thank Shri K. C. Reddy, Hon'ble Minister for Production, for having taken a keen interest in developing this plant. I can take you in confidence to tell you that Government have invited a team of Soviet experts to advise it to put up a fully integrated drug industry with its own basic and intermediate chemical plants to make all essential drugs in our country. It is very likely that as a part of this new scheme, this plant may before long be producing streptomycin, biomycin and other needed tetracyclines and the latest, most potent and least toxic Soviet antibiotic, Albo-mycin, and vitamins B<sub>2</sub> and B<sub>12</sub>. The Soviet experts have already suggested



how to practically double the production of this plant to about 35 million mega units per year without incurring much additional cost.

I must not be understood to be unmindful of the tremendous work our pharmaceutical industry in the private sector is doing in spite of the fact that they are working under great handicaps. The country does not produce the intermediate chemicals and not even all the basic chemicals needed for the manufacture of essential drugs. It is forced to make drugs from imported penultimate products or purify almost ready-made imported drugs. This activity does not give our scientists and technicians any opportunity to show what they can do. Then again the cost of drugs so made remains practically the same as that of the imported product. This works to the great disadvantage of our people who are poor and hinders Government in their work of expansion of medical relief to the people. I am, therefore, glad and you will share my pleasure when I tell you that Government are actively working to make the country self-sufficient as regards basic chemicals, intermediates and all the essential drugs. The new industry which the Government propose to put up with the help of Soviet experts will be able to help private industry also by providing them with intermediates, etc. to enable them also to make drugs if they choose to do so. Then a healthy rivalry will be created between state and private plants to excel each other.

Before I close I should like to touch on another point of some importance, that is, the relationship of research to production. In this connection I should like to begin by quoting Professor G. F. Powel, Nobel Laureate, Bristol University and Chairman, World Federation of Scientific Workers:

“In all our work, my colleagues and I have received inspiration, even when we were least conscious of it, from those great aims of natural philosophy which were embodied in the doctrine of utility and progress, so clearly enunciated by my great countryman, Francis Bacon, for the view that the true end of science is to lighten the burden of labour and to enrich human life.”

I feel that at the present stage of development of our country, research and production must be intimately linked. Research should start with the solution of problems that face the country. In the implementation of such research projects, fundamental or so-called pure science will find its own place. All our laboratories, including perhaps the universities, should be closely attached to production plants. In the modern world there is no distinction between pure and applied science. The work should begin from a practical problem and no one should consider himself too superior to solve problems facing the country. Beginning from there science can rise to any heights. Our laboratories should have plenty of staff and equipment to attend to both immediate and fundamental problems at the same time. As the Chairman of the Pharmaceuticals & Drugs Research Committee, I and my colleagues are very conscious of the fact that at present we spend our money and effort on researches into finding improvements on known drugs, when we hardly make any essential drugs at all in the country. We must remember we are a poor people ; all our talent and our resources should go in the first instance to

making the essential drugs we require. Then we will not only do a great service to our people, but would give a base to our scientists from which they would be able to work on a level of equality with the scientists of the rest of the world and endeavour to outshine them. Fortunately, we have excellent talent and we should provide them with an opportunity to show what it can do.

## *Vote of Thanks*

DR. V. SUBRAHMANYAN

Director, Central Food Technological Research Institute, Mysore  
and Chairman, Biochemical Research Committee, CSIR

To me, it is much more than the conventional vote of thanks to address you on this occasion. My association with you, Sir, is much closer than the fortuitous feature of meeting through the symposium. Ours is a life-long association through the medium of the national institution which you have helped to locate in Mysore. Today, I consider it a doubly great privilege to say how thankful we are for your having spared the time to come to inaugurate the symposium.

Looking back from those hard and trying forties when we were endeavouring to make penicillin on a laboratory scale, it is a thrilling experience to be in the home of antibiotics today. The *Hindustan Antibiotics (Private) Ltd.* is a great achievement which we can all be proud of. In the early days we were making miserably small quantities on a laboratory scale, but even that brought us a thrill. We used to get hundreds of telegrams not only from within this country but also from Ceylon, Burma and Afghanistan, begging for penicillin for cases for which there was no other help. Many of them were late cases, but in a few the results were dramatic and we got very grateful messages from the people who were saved. It was this experience which led the late Dr. Bhatnagar and the Pharmaceuticals & Drugs Research Committee to provide generously for antibiotics research in Bangalore. We also had the enthusiastic collaboration of a leading industrialist who was keen on starting production. Subsequently, there was a special meeting in Bombay which was attended by the late Dr. Bhatnagar, Maj.-Gen. Sokhey, Dr. Jivraj Mehta and myself which led to a decision to establish the antibiotics plant under national auspices. The decision disappointed some people, but judging from the results achieved, there will be no longer any cause for regret.

The idea of a symposium on antibiotics to be held at Pimpri first came from my friend Dr. Shrivastava, who is a member of the Steering Committee, and he is here with us today. To him and to my colleagues Drs. Sreenivasan and Ganapathi, who had to work very hard, our warm



thanks are due. There is also my friend Dr. Bhatia who had to do a tremendous amount of work in connection with the organization of the symposium. The success of our effort was possible chiefly because of the enthusiastic response we had from Col. Dogra and his assistants. Without making any distinction, I may state that our thanks are due to one and all of them. No effort of this type would have assumed proper shape unless we got adequate collaboration from our colleagues who have participated in the symposium and who have contributed to the discussions. To them and also to the representatives of the various international organizations who have very generously responded to our call, our thanks are also due. To one and all of you and also to several delegates who have come from different parts of the country, I express our very warm thanks and appreciation. We have done our best to make the symposium a success. If there is a lapse in any direction, I would request you to forgive us. We are all together in it.





## Distribution of Higher Myxobacteria in Indian Soils

B. N. SINGH, S. MATHEW & M. SREENIVASAYA  
Central Drug Research Institute, Lucknow

A systematic survey of the occurrence of higher myxobacteria in soils around Lucknow has been made. *Polyangium fuscum*, *Chondromyces* sp. and *Myxococcus fulvus* seem to be commonly present, while *Myxococcus virescens* and *Chondrococcus exiguus* have been recorded only from a few soil samples. Strains of *P. fuscum* and *Chondromyces crocatus* grew best at 37° and 30°C. respectively on solid media. The optimum temperature for the growth and production of fruiting bodies in strains of *M. fulvus* and *Myxococcus* sp. has been found to be 33°C. Six strains belonging to *M. fulvus* and *P. fuscum* have been tested on a number of human pathogenic bacteria, like dysentery, cholera and typhoid, by a method developed for the purpose. A distinct strain variation of lytic action towards the bacteria tested, as judged by the area of lysis, as well as a great variation in their lytic capacity towards a given test organism have been observed.

Myxobacteria were recognized by Thaxter<sup>1</sup> as an independent group. The earlier workers considered them to be dung organisms because they isolated them from dung of various animals that had laid on the ground for sometime. The distribution of myxobacteria in different types of soil has hitherto attracted little attention. So far no survey of myxobacteria in Indian soils has been made, although these organisms have been shown to be common soil inhabitants by Krzemieniewskis<sup>2</sup> in Polish, Beebe<sup>3</sup> in American, Singh<sup>4</sup> and Noren<sup>5</sup> in British and Noren<sup>6</sup> in Swedish soils. Myxobacteria constitute an interesting group of micro-organisms which offer a fertile field for exploring the possibility of discovering new antibiotic factors, especially against Gram-negative bacteria. The lysis of both the dead and living Gram-positive and Gram-negative bacteria have been studied by several workers<sup>4,7,8</sup>. Oxford and Singh<sup>9</sup>, Oxford<sup>10</sup> and Noren<sup>8,11,12</sup> have shown that certain strains of myxobacteria growing on cell-free medium produce two substances, extracellular lytic (proteolytic) enzymes and antibiotic. Very recently Mathew and Dudani<sup>13</sup> showed that *Myxococcus virescens* and *M. fulvus* were able to lyse dysentery, cholera and typhoid groups of organisms to varying degrees on solid media.

The distribution of myxobacteria in soils around Lucknow and the bacteriolytic properties of certain strains towards human pathogenic bacteria have been described in this paper.

#### METHODS OF ISOLATION AND CULTURE

Krzemieniewskis's methods<sup>2</sup> as modified by Beebe<sup>3</sup> and Singh<sup>4</sup> were followed in these investigations for obtaining myxobacteria from soils. In the first method fresh sterilized rabbit dung from domesticated rabbits, and in the second, young nutrient agar slope cultures (2-5 days old) of *Aerobacter* sp., strain 1912 and *Bacterium coli* were used. Fruiting bodies developing either on sterilized rabbit dung or on non-nutrient agar supplied with a species of bacteria were transferred to streaks of *Aerobacter* sp. on non-nutrient agar plates<sup>4</sup> and incubated at 25°-30°C. Fruiting bodies or a portion of the swarm of myxobacteria free from contaminating amoebae and other micro-organisms were inoculated on fresh *Aerobacter* sp. streaks in order to get 'pure mixed' cultures of myxobacteria growing and lysing bacteria.

#### Distribution of myxobacteria in soil

Fifty samples consisting of heavily manured and unmanured field, garden and grassland soils from around Lucknow were examined. The samples were taken from the top six inches of the soil. Almost every soil sample was found to contain myxobacteria. *Polyangium fuscum*, *Chondromyces crocatus*, *Chondromyces* sp., *Myxococcus fulvus*, *M. virescens* and *Chondrococcus exiguus* are the species which have so far been recorded. *P. fuscum*, *Chondromyces* sp. and *M. fulvus* seem to be very commonly present. The distribution of the common species in Lucknow soils seems to be different from British<sup>4,5</sup> and American<sup>3</sup> soils. Species of *Myxococcaceae* were easily detected in soil by Singh's<sup>4</sup> method while Krzemieniewskis<sup>2</sup>-Beebe<sup>3</sup> method gave disappointing results. This suggests that rabbit dung pellets are unfavourable for the growth of lytic myxobacteria. Similar results were obtained by Singh<sup>4</sup> and Noren<sup>5</sup> in British soils. *P. fuscum* and *C. crocatus* appeared more often on rabbit dung<sup>3</sup> than on non-nutrient agar plates<sup>4</sup> supplied with a species of bacteria. These species may not have been abundant enough to occur in 0.5 g. of soil used in Singh's<sup>4</sup> method or some strains may not be lytic or may not attack the bacteria used in the experiments.

#### Influence of temperature on growth of myxobacteria

The influence of temperature on the growth of species of myxobacteria have been found to be different by different workers<sup>8</sup>. In the present work *P. fuscum* (strains 1, 10, 11 and 13), *C. crocatus* (strain 19), *M. fulvus* (strains 1 and 16) and *Myxococcus* sp. (strains 12, 13 and 20) were grown on non-nutrient agar with *Aerobacter* sp. at 25°, 30° and 37°C. *P. fuscum* grew best at 37°C. but fruiting bodies were more numerous at 33°C. The growth and production of fruiting bodies were best at 30°C. in the case of *C. crocatus* and at 37°C., it did not grow. *M. fulvus* and other strains of *Myxococcus* sp. grew best at 33°C. and produced large number of fruiting bodies.



### Lysis of human pathogenic bacteria by myxobacteria

Petri dishes, each containing four glass rings (2.5 cm. internal diam., 1 cm. depth and 1-2 mm. thickness) were sterilized and 2.5 per cent non-nutrient agar of pH 7.2 was poured as a layer. After the agar solidified, the glass rings were removed and the circular cavities were filled with nutrient agar of pH 7.2. Thick suspension of a young culture (2 days old on nutrient agar slope) of a test bacterium in 0.5 per cent sodium chloride solution was spread as a thin layer on the circular nutrient agar surfaces and incubated at 37°C. for 48 hr. At the centre of the two bacterial patches in a Petri dish, a small loopful of fruiting bodies of a myxobacteria to be tested was inoculated and the other two were left as controls. The plates were incubated at 37°C. and readings of the cleared lysis zone was measured at 24-hr. intervals for 4 days. The area of cleared zones (average of two measurements) after 4 days has been expressed as mm<sup>2</sup>.

Table 1 shows the lysis of bacterial strains by *Myxococcus fulvus* (strains 1 and 16) and *Polyangium fuscum* (strains 1, 10, 11 and 13). *Bacillus subtilis* and *Mycobacterium phlei* are not lysed by any strains of myxobacteria tested while *Vibrio cholerae* rough 56913 and *V. cholerae* Ogawa 70153 are only slightly lysed by certain strains. The two strains of *M. fulvus* are similar in their lytic action towards the bacteria tested but they differ in their potency towards different bacteria as judged by the areas of lysed zones. In the case of *P. fuscum* the strain differences are much more marked. Strain 13 is unable to lyse *Shigella paradysenteriae* Flexner and *Salmonella paratyphosa* A while the other three strains lyse these bacteria to varying degrees. As in the case

TABLE 1—LYSIS OF BACTERIAL STRAINS OF MYXOBACTERIA  
(Area of clearance expressed in mm<sup>2</sup>. after a period of 4 days)

BACTERIAL STRAINS TESTED	<i>M. fulvus</i> strain 1	<i>M. fulvus</i> strain 16	<i>P. fuscum</i> strain 1	<i>P. fuscum</i> strain 10	<i>P. fuscum</i> strain 11	<i>P. fuscum</i> strain 13
<i>Staphylococcus aureus</i>	58	18	531	576	229	456
<i>Bacillus subtilis</i>	nil	nil	nil	nil	nil	nil
<i>Mycobacterium phlei</i>	nil	nil	nil	nil	nil	nil
<i>Shigella paradysenteriae</i> Flexner	46	46	445	420	53	nil
<i>Vibrio cholerae</i> Rough 56913	nil	nil	nil	66	nil	nil
<i>Vibrio cholerae</i> Ogawa 70153	21	47	nil	nil	nil	nil
<i>Salmonella paratyphosa</i> A	206	121	441	395	260	nil
<i>S. paratyphosa</i> B	272	399	369	289	130	134
<i>Proteus</i> X19	37	93	400	201	93	250
<i>Escherichia coli</i>	148	126	454	469	42	507
<i>Aerobacter</i> sp. strain 1912	169	306	132	350	..	47

of *M. fulvus*, the areas of lysed zones produced on different bacteria by strains of *P. fuscum* differ markedly.

*M. fulvus* (strains 1 and 16) and *P. fuscum* (strains 1, 10 and 11) grew to some extent in the presence of *M. phlei* without showing any apparent lysis.

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# Screening of Indian Soils for Antibiotic- and Vitamin B<sub>12</sub>-producing Micro-organisms

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A large number of actinomycetes isolated from soil samples collected from different parts of the country have been studied for their antibiotic- and vitamin B<sub>12</sub>-producing properties.

A systematic screening of soil samples from different parts of the country for antibiotic-producing actinomycetes has been made and the results have been recorded and discussed in this paper.

## EXPERIMENTAL

Subsurface soil samples from different parts of the country were plated out on Raulin Thom<sup>1</sup> and Conn's agar<sup>2</sup> for the isolation of fungi and actinomycetes. The plates were incubated at 24°C. for fungi, and 28°-30°C. for actinomycetes, for 6-7 days. The fungi and colonies of actinomycetes were picked out on the basis of their pigment difference and other morphological characteristics, and further purified.

## Screening

In order to be able to screen a large number of organisms it was desirable to have a rapid and sensitive method capable of showing not only the presence but also the extent of antibacterial activity. For this purpose, Waksman's cross streak method<sup>3</sup> was followed. Each fungal isolate was individually transferred to the centre of a Petri dish containing Wickerham's agar and incubated for 6-8 days. At the end of this period six test organisms—*Staphylococcus aureus*, *Escherichia coli*, *Shigella shigae*, *Shigella flexneri*, *Salmonella typhi*, *Vibrio cholerae*—were radially streaked around the mould colony and incubated overnight at 37°C. Next day the distance between the periphery of the mould or actinomycete culture and the edge of growth of the test organism was measured and taken as the zone of inhibition against that particular organism. The cultures with appreciable zones of inhibition against more than one or two test organisms were grown on several liquid media and the antibacterial activity of the culture filtrates was tested by the cup-plate method against the same set of test organisms.

The actinomycete cultures isolated were simultaneously tested for vitamin B<sub>12</sub> activity by inoculating individual colonies on plates containing the appropriate agar medium seeded with Davis and Mingioli's vitamin B<sub>12</sub> dependent *E. coli* mutant (113-3)<sup>4</sup>. *E. coli* exhibition zones observed around any actinomycete colony were taken as a positive indication of the presence of vitamin B<sub>12</sub><sup>5</sup>. After preliminary screening, actinomycetes with positive vitamin B<sub>12</sub> activity were grown on a suitable liquid medium (consisting of glucose, calcium carbonate and few other supplements like distiller's solubles, peptone, etc.) and the B<sub>12</sub> activity of the culture filtrate and the mycelium was assayed with *Lactobacillus leichmannii*<sup>6</sup>, *E. coli* mutant(113-3)<sup>7</sup> and *Ochromonas malhamensis*<sup>8</sup> by the serial dilution method.

### RESULTS AND DISCUSSION

Four hundred and twenty fungal cultures were isolated from eighty soil samples out of which 236 were tested by primary screening. Nearly 66 per cent of the fungal isolates had varying amounts of activity against one or more of the test organisms (Table 1). Two hundred and forty cultures were selected for secondary screening on the basis of primary screening results out of which nearly 75 per cent were found to be active against one or more of the test organisms (Table 2).

TABLE 1—PRIMARY SCREENING OF FUNGAL CULTURES

NO. OF TEST ORGANISMS AGAINST WHICH THE ISOLATES ARE ACTIVE	NO. OF CULTURES	PERCENTAGE OF TOTAL NO. OF CULTURES
0	81	34.0
1	37	15.6
2	24	10.2
3	18	7.9
4	23	9.9
5	10	4.0
6	43	18.0

TABLE 2—SCREENING OF CULTURES BY CUP-PLATE TECHNIQUE

NO. OF TEST ORGANISMS AGAINST WHICH THE ISOLATES ARE ACTIVE	NO. OF CULTURES	PERCENTAGE OF TOTAL NO. OF CULTURES
0	59	24.6
1	53	22.1
2	49	20.4
3	42	17.5
4	12	5.0
5	17	7.1
6	8	3.3



TABLE 3—COMPOSITION (%) OF LIQUID MEDIA USED FOR ISOLATION OF ACTINOMYCETES

## LIQUID MEDIA

	I	II	III	IV	V	VI	VII	VIII	IX	X	XI
Glucose	2.5	1.0	2.5	1.0	1.0	1.0	..	0.25	1.0	1.0	1.5
Peptone*	..	..	..	0.5	0.5	..	..	..	0.5	0.5	..
Tryptone or Proteose peptone*	..	..	..	..	0.3	..	..	..	..	0.3	..
Meat extract	..	..	..	0.3	..	0.3	..	..	0.3	..	..
Yeast extract	0.25	..	0.5	..	..	..	1.0	0.025	..	..	..
Sodium chloride	..	0.5	0.25	0.5	0.5	0.5	0.5	..	0.5	0.5	0.5
Papain broth	..	..	..	..	..	..	..	..	..	..	..
Starch	..	..	..	..	..	..	1.0	..	..	..	..
Soyabean meal or soyabean meal digest <sup>1</sup> , cc.	2.5	1.0	4.0	..	..	1.0	..	2.5	0.5	0.5	..
Casein hydrolysate <sup>2</sup> , c.c.	..	..	..	..	..	10	..	20	..	..	..
Corn-steep liquor <sup>3</sup>	..	..	..	..	..	10	..	..	..	..	..
Potassium chloride	0.4	..	..	..	..	..	..	..	..	..	3.0
Calcium carbonate	0.8	0.1	..	..	..	..	..	0.04	..	..	..
Ammonium sulphate	0.5	..	..	..	..	..	..	0.08	..	..	0.3
Potassium phosphate (K <sub>2</sub> HPO <sub>4</sub> )	0.02	..	..	..	..	..	..	0.05	..	..	..
Ferrous sulphate (FeSO <sub>4</sub> ·7H <sub>2</sub> O)	..	..	..	..	..	0.2	0.2	0.04	..	..	..
Ammonium phosphate [(NH <sub>4</sub> ) <sub>2</sub> HPO <sub>4</sub> ]	..	..	..	..	0.001	0.002	0.002	..	..	..	..
Magnesium sulphate (MgSO <sub>4</sub> ·7H <sub>2</sub> O)	..	..	..	..	..	0.4	..	..	..	..	..
Calcium chloride	..	..	..	..	..	0.1	..	..	..	..	..
Zinc sulphate (ZnSO <sub>4</sub> ·7H <sub>2</sub> O)	..	..	..	..	..	0.04	..	..	..	..	..
	..	..	..	..	..	0.001	..	..	..	..	..

\* Difco

1. 25 g. defatted soyabean meal + 200 cc. water (distd.) + 1.5 g. papain incubated for 3 hr. at 45°C., heat, filter and made up to 200 cc.

2. Prepared by hydrolysis with hydrochloric acid.

3. Prepared by treatment of corn with sodium bisulphite and made up as a 10% solution.

Most of the saprophytic fungi isolated were either *Aspergillus* or *Penicillium* type. Twenty of these moulds were investigated for the isolation of their active principles. Kojic acid was obtained from sixteen of these while three others yielded citrinin<sup>9</sup>. The remaining one identified as a non-pigmented variant of *Penicillium notatum* Westling by Prof. S. R. Bose of Calcutta (culture No. 29C) yielded an antibiotic (X 29C) unlike penicillin, notatin or notalysin<sup>10</sup>. This antibiotic was active against a number of test organisms but was found to be highly toxic. When administered intravenously in mice LD<sub>0</sub> and LD<sub>100</sub> were found to be 37 and 74 mg. respectively per kg. body weight. It was, therefore, tried for topical application in experimental ulcers in rabbits and the results were found to compare favourably with other well-known antibiotics<sup>11</sup>.

In view of the fact that except penicillin, majority of the therapeutically useful antibiotics have been isolated from actinomycetes, more emphasis was laid on screening soils for actinomycetes.

Nine hundred and seventy-three cultures of actinomycetes have so far been isolated from 188 soil samples and screened for their antibacterial activity as well as for the presence of vitamin B<sub>12</sub>. The cultures which were found promising on rapid screening were grown on several liquid media (Table 3) as the actinomycetes are known to be very fastidious in their growth requirements.

A summary of the data regarding location of soil samples, number of actinomycetes isolated and their antibacterial activity on rapid screening and secondary screening by cup-plate assay is presented in Table 4.

The results of primary screening (Table 5) showed that nearly 40 per cent of the isolates were active against one or more of the test organisms. It also showed that the largest number of cultures were active against *S. aureus*, the next best activity was noted against *V. cholerae* while the least number of cultures showed activity against the Gram-negative test organisms.

Only 24 per cent of the actinomycetes, found active on rapid screening, reproduced the activity when grown in liquid media as assayed by the cup-

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TABLE 4—SCREENING OF ACTINOMYCETES

LOCATION	NO. OF SOIL SAMPLES	NO. OF CULTURES ISOLATED	NO. ACTIVE IN RAPID SCREENING	NO. GROWN IN LIQUID MEDIA	NO. ACTIVE IN LIQUID MEDIA
Simla	17	72	39	33	6
Baroda	12	73	26	6	1
Kasauli	15	116	24	6	3
South India	13	45	27	20	2
Lucknow City	37	175	57	28	4
Lucknow dist.	75	318	136	63	31

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TABLE 5—NUMBER OF ACTINOMYCETES ACTIVE AGAINST VARIOUS TEST ORGANISMS

TEST ORGANISM	ZONES OF INHIBITION (MM.)					TOTAL NO. TESTED
	0	10	10-20	20-30	30	
<i>S. aureus</i>	72	32	101	68	38	311
<i>E. coli</i>	177	32	45	34	23	311
<i>Shigella shigae</i>	188	15	29	48	31	311
<i>Shigella flexneri</i>	192	27	47	35	10	311
<i>S. typhi</i>	191	15	34	57	14	311
<i>V. cholerae</i>	157	41	39	54	20	311

TABLE 6—ACTINOMYCETES ACTIVE WHEN GROWN IN LIQUID MEDIUM

TEST ORGANISM	ZONES OF INHIBITION (MM.)			TOTAL NO. TESTED
	0	10-20	20	
<i>S. aureus</i>	127	26	14	167
<i>E. coli</i>	143	22	2	167
<i>Shigella shigae</i>	135	27	5	167
<i>Shigella flexneri</i>	143	23	1	167
<i>S. typhi</i>	140	22	5	167
<i>V. cholerae</i>	127	29	11	167

plate method (Table 6). Reasons for this non-reproducibility of activity has been discussed by various authors<sup>2</sup>.

The actinomycetes which were isolated seem to be poor producers of vitamin B<sub>12</sub>. None of them produces vitamin B<sub>12</sub> in quantities comparable with those which are being commercially exploited.

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# Screening of Soils for the Isolation of Antagonistic Organisms by the Soil Plaque Technique

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Antagonistic activities of different physiological groups of microbes have been demonstrated on the soil surface. Enrichment of soil plaques with starch, mannitol, sucrose and different media, viz., nutrient broth, potato dextrose solution, and Czapek's solution for the direct demonstration of inhibition zone on the soil surface have been investigated. The possibility of utilizing the soil plaque technique for screening of soil samples for the isolation of antagonistic organisms has been indicated.

Soil is a complex biological system in which the qualitative and quantitative compositions of the heterogeneous population are in a dynamic state of equilibrium. The numerous associations among the soil micro-organisms that exist exhibit relationships that are either mutualistic or antagonistic to each other. The study on the associative and antagonistic inter-relationships among micro-organisms, particularly that which has been directed to the production of antibiotic substances, has been by indirect methods. Though the enrichment method for isolating different physiological groups of microbes had its beginning in 1926, when Winogradsky<sup>1</sup> demonstrated the method of isolating *Azotobacter* from soil, the soil enrichment technique for the isolation of different physiological groups of organisms has not received due attention. While employing the soil plaque technique for the isolation of *Azotobacter*, Majumder and Sen<sup>2</sup> encountered an inhibition zone of the soil surface. This suggested the possibility of isolating antagonistic organisms directly from the soil surface<sup>3</sup> and indicated a need to study the phenomenon of antagonism on the natural habitat.

The use of enriched soil plaque technique for the isolation of antagonistic organisms required a knowledge of the physiological groups of microbes that are likely to grow in preference to the others with a particular nutrient or combination of nutrients. Investigations in this direction would throw light on the inhibitory actions of different species to each other at particular nutritional and ecological conditions. The authors have endeavoured to elucidate in this paper, some of the points which are necessarily to be followed

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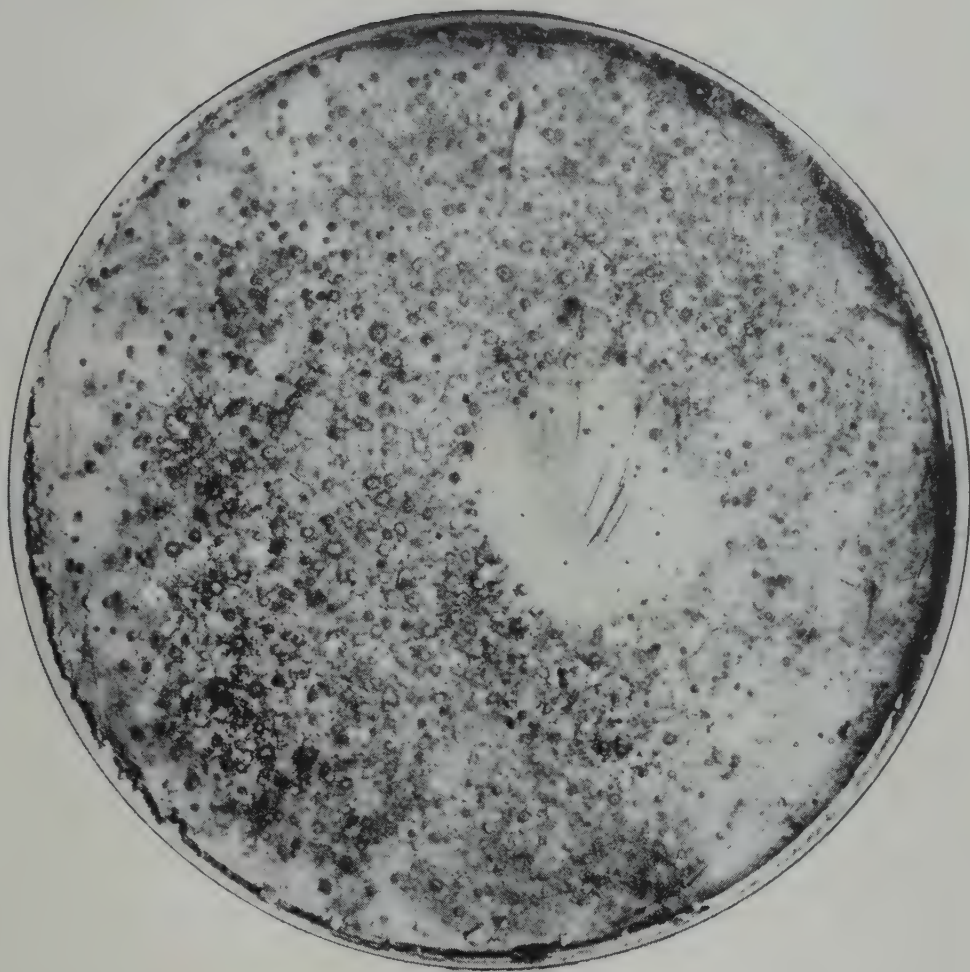


FIG. 1—ENRICHED SOIL PLAQUE (WITH 5% STARCH) SHOWING ANTAGONISTIC ZONE (CHINSURAH SOIL)

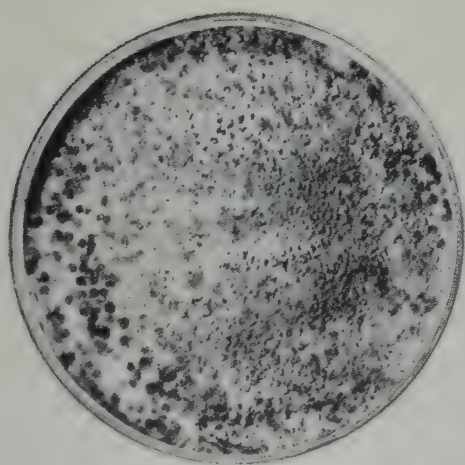


FIG. 2—ENRICHED SOIL PLAQUE (WITH 5% STARCH) SHOWING MICROBIAL COLONIES, BUT NO ANTAGONISTIC ZONE (CHINSURAH SOIL)

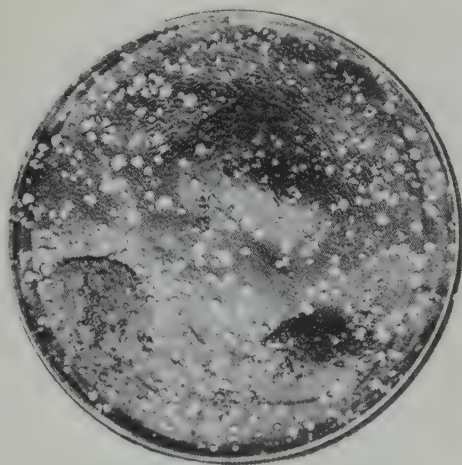


FIG. 3—ENRICHED SOIL PLAQUE (WITH 10% STARCH) SHOWING COLONIES OF AZOTOBACTER ONLY (KHARAGPUR, FODDER FIELD SOIL)





for the use of enriched soil plaque technique for the isolation of antagonistic micro-organisms.

### EXPERIMENTAL

Soil samples for the studies were collected from Chinsurah (alluvial), Gosaba (saline) and Kharagpur (red lateritic). These were well mixed, air dried and sieved.

The soil plaques were prepared by adding water or solution as the case may be to a weighed quantity of soil so as to bring them to the consistency of a thick paste with "sticky point". Excess moisture was avoided. The paste was transferred to Petri dishes, pressed well and surface smoothened with a wet glass slide. Where enriched soil plaques were prepared, starch or sucrose or mannitol in requisite quantities was mixed with soils prior to the addition of water. For studying the effects of enriching the soil with media, plaques were prepared with nutrient broth or potato dextrose solution or Czapek's solution<sup>4</sup> in place of water. The soil plaques were incubated at 30°C. and noted for growth for seven days. Soil plaques were also prepared to examine the effects of ammonium sulphate and sodium nitrate in combination with starch.

After incubation, microbial colonies on the soil surface were examined and the presence of inhibition zone noted. The isolation and identification were carried out by usual microbiological methods<sup>5-9</sup>. The organisms present in the inhibition zone were isolated and tested for their antibiotic activities by agar plate method. The results are presented in Table 1.

### DISCUSSION

Predominance of a particular species of organism in soil is due to the preferential biotic and ecological conditions. A nitrilite at a particular level upsets the equilibrium of the normal biological complex. Thus the biotic association between groups tends to change from one to the other. The results presented in this paper demonstrated that addition of nitrilites (starch, sucrose) and media (nutrient broth, Czapek's solution and potato dextrose solution) change the population and help a few species to grow faster or even to the extent of elaboration of antagonistic principle *in situ* in preference to others. This indicates that the various degrees of associations, of which antagonism is one, can be exhibited by changing the nutritional status of the soil and thereby helping the isolation of physiological groups by obtaining accelerated visual growths on the soil surface. Soil enrichment with starch helped *Azotobacter* and other amylolytic organisms to grow on the soil (Figs. 1-3). That is why the inhibition of *Azotobacter* could be demonstrated on the soil plaque (Fig. 1). The growths of *Bacillus polymyxa* and *Pseudomonas fluorescens*, the antagonists, however, were not visible on the soil surface, but on plating with nutrient agar the predominance of these at the inhibition zone could be detected.

It was expected that the microbial count of the soils which have been drawn

TABLE 1—EFFECTS OF DIFFERENT ENRICHMENTS ON GROWTH OF MICROBES ON SOIL PLAQUES

SOIL SAMPLE	ENRICHMENT INGREDIENT G./100 G. SOIL	NO. OF SAMPLES TESTED	ANTAGO- NISTIC ZONE (NO.)	ORGANISMS PREDOMINAT- ING ON THE SOIL SURFACE	ANTAGONISTS
Chinsurah	<i>Starch</i>				
	0	3	..	..	..
	5	3	+(1)	Azotobacter & greenish fungal colonies	<i>Pseudomonas fluorescens</i>
	10	3	+(1)	<i>Azotobacter</i>	<i>Bacillus polymyxa</i>
Gosaba	15	3	..	do.	..
	0	5	..	..	..
	5	5	+(1)	Fungus (unidentified)	Bacterium (unidentified)
	10	5	..	..	..
Kharagpur Fodder field	0	5	..	..	..
	5	5	+(1)	<i>Azotobacter</i>	Bacterium (unidentified)
	10	5	..	do.	..
	0	5	..	..	..
Railway farm vegetable plots	0	5	..	..	..
	5	5	+(2)	<i>Azotobacter</i>	<i>Bacillus polymyxa</i> and <i>Pseudomonas fluorescens</i>
	10	5	..	do.	..
	0	5	..	..	..
Nursery soil I.I.T.	10	5	..	do.	..
	0	5	..	..	..



	5	5	..	No surface growth, but swelling or blisters were noted	..
	10	5	..	<i>Azotobacter</i>	..
Flower bed	0	5	..	..	..
I.I.T.	5	5	..	..	..
	10	5	..	..	..
Kharagpur Fodder field	Sucrose 0	5	..	..	..
	2.5	5	..	<i>Azotobacter</i> and yeasts	..
	5	5	..	Yeasts and <i>Cunninghamella</i>	..
	10	5	..	Yeasts	..
	15	5	..	Yeasts	..
Railway farm	0	5	..	..	..
vegetable plots	2.5	5	..	<i>Azotobacter</i> and yeasts	..
	5	5	..	<i>Cunninghamella</i> and yeasts	..
	10	5	..	Yeasts	..
Nursery soil	0	5	..	..	..
	2.5	5	..	Yeasts	..
	5	5	..	Yeasts	..
	10	5	..	Yeasts	..

Contd.

SOIL SAMPLE	ENRICHMENT INGREDIENT G./100 G. SOIL	NO. OF SAMPLES TESTED	ANTAGO- NISTIC ZONE (NO.)	ORGANISMS PREDOMINAT- ING ON THE SOIL SURFACE	ANTAGONISTS
Flower bed	<i>Starch</i> 0	5	..	..	..
	2.5	5	..	Yeasts	..
	5	5	..	Yeasts	
	10	5	..	Yeasts	..
Fodder field	<i>Mannitol</i> 0	3	..	..	..
	2	3	..	..	..
	5	3	..	..	..
	0	3	..	..	..
Railway farm	2	3	..	..	..
	5	3	..	..	..
	<i>Starch</i> 5 + 1 cc. 1% (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	5	..	Different types of fungi	..
	5 + 1 cc. 2% (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	5	+ (1) (Fig. 4)	do.	Could not be isolated
Kharagpur Fodder field	5 + 1 cc. 1% NaNO <sub>3</sub>	5	..	No growth	..
	5 + 1 cc. 2% NaNO <sub>3</sub>	5	..	do.	..
	10 + 1 cc. 1% (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	5	..	Different types of fungi and bacteria	..

	10 + 1 cc. 2% $(\text{NH}_4)_2\text{SO}_4$	5	..	do.	..
	10 + 1 cc. 1% $\text{NaNO}_3$	5	..	No growth	..
	10 + 1 cc. 2% $\text{NaNO}_3$	5	..	do.	..
Gosaba	5 + 1 cc. 1% $(\text{NH}_4)_2\text{SO}_4$	5	..	Various fungi	..
saline	5 + 1 cc. 2% $(\text{NH}_4)_2\text{SO}_4$	5	..	do.	..
soil	5 + 1 cc. 1% $\text{NaNO}_3$	5	..	Actinomycetes	..
	5 + 1 cc. 2% $\text{NaNO}_3$	5	..	Actinomycetes & <i>Neocosmospora</i>	<i>Neocosmospora</i>
	<i>Nutrient broth</i>				
Kharagpur Fodder field	do.	3	..	Numerous types of bacteria	..
Railway farm	do.	3	..	do.	..
Nursery	do.	3	..	do.	..
	<i>Potato-dextrose solution</i>				
Kharagpur Fodder field	do.	3	..	Fungi and bacteria	..
Railway farm	do.	3	..	do.	..
Nursery	do.	3	..	do.	..
	<i>Czapek's solution</i>				
Kharagpur Fodder field	1 Normal	5	..	<i>Cunninghamella</i>	..
	2.5 Normal	5	..	<i>Trichoderma</i> and <i>Verticillium</i>	..
	5 Normal	5	..	..	..

Contd.



SOIL SAMPLE	ENRICHMENT INGREDIENT G./100 G. SOIL	NO. OF SAMPLES TESTED	ANTAGO- NISTIC ZONE (NO.)	ORGANISMS PREDOMINAT- ING ON THE SOIL SURFACE	ANTAGONISTS
<i>Starch</i>					
Railway farm	1 do.	5	..	<i>Cunninghamella</i>	..
	2.5 do.	5	..	<i>Trichoderma</i> , <i>Verticillium</i> <i>strum</i> and <i>Penicillium</i>	..
	5 do.	5	..	<i>Verticillium</i> and <i>Peni- cillium</i>	..
Chinsurah	1 do.	5	..	<i>Cunninghamella</i>	..
	2.5 do.	5	..	<i>Trichoderma</i> and <i>Verti- cillium</i>	..
	5 do.	5	..	<i>Penicillium</i> and <i>Asper- gillus</i>	
	1 do.	5	..	..	
Kharagpur Nursery	2.5 do.	5	..	<i>Verticillium</i> , <i>Verti- cillium</i> and <i>Tricho- derma</i>	
	5 do.	5	..	<i>Penicillium</i>	..

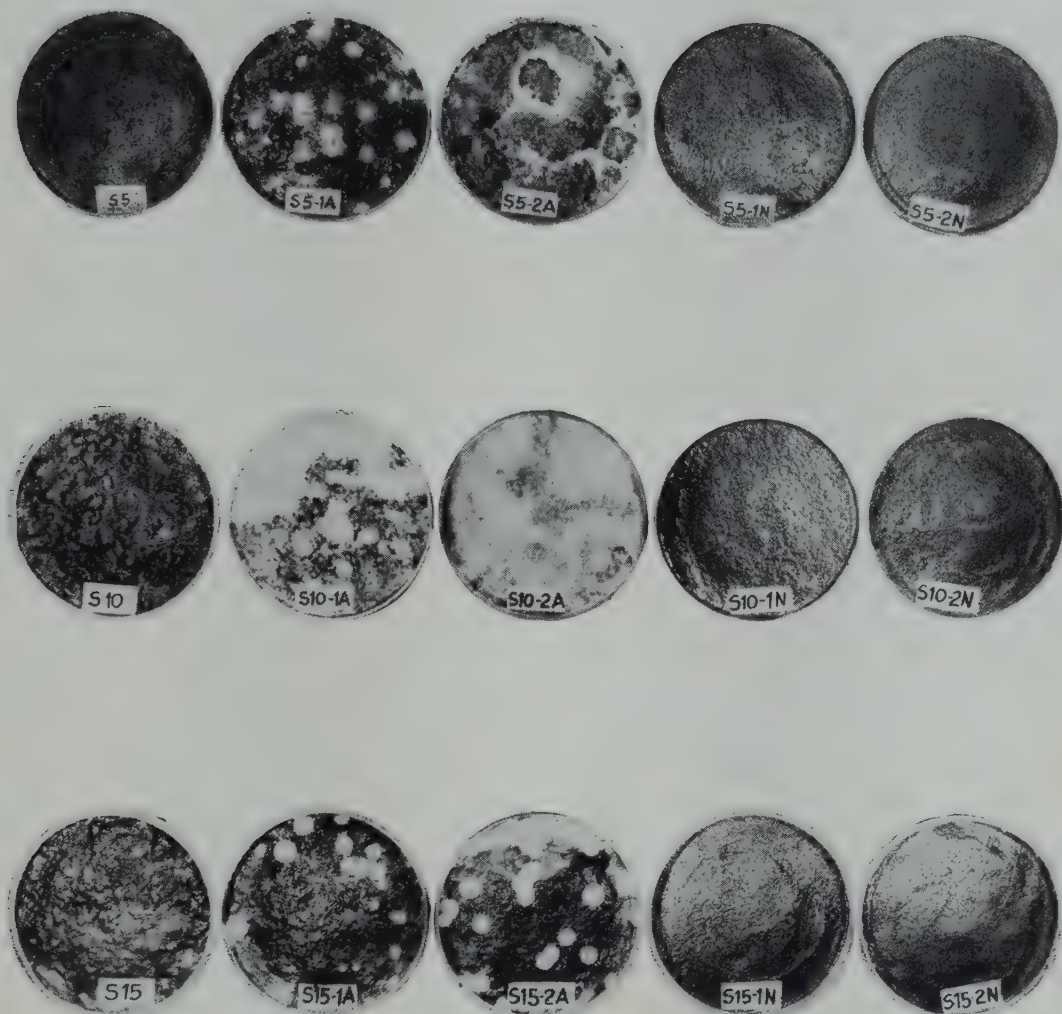
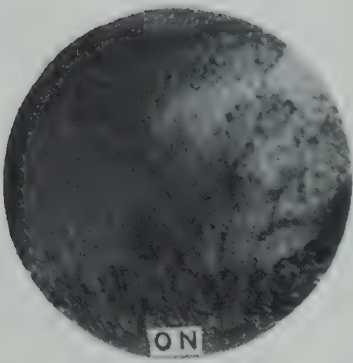
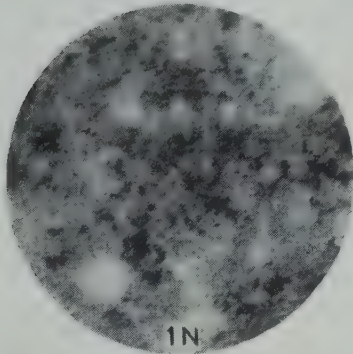


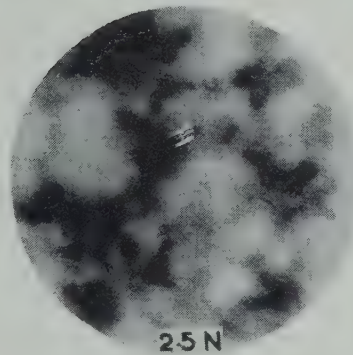
FIG. 4—EFFECT OF ENRICHMENT WITH AMMONIUM SULPHATE AND SODIUM NITRATE ON SOIL STARCH PLATES (KHARAGPUR, FODDER FIELD SOIL) [S5, STARCH 5% ; S5-1A, STARCH 5% + AMMONIUM SULPHATE 1% ; S5-2A, STARCH 5% + AMMONIUM SULPHATE 2% ; S5-1N, STARCH 5% + SODIUM NITRATE 1% ; S5-2N, STARCH 5% + SODIUM NITRATE 2% ; S10, STARCH 10% ; S10-1A, STARCH 10% + AMMONIUM SULPHATE 1% ; S10-2A, STARCH 10% + AMMONIUM SULPHATE 2% ; S10-1N, STARCH 10% + SODIUM NITRATE 1% ; S10-2N, STARCH 10% + SODIUM NITRATE 2% ; S15, STARCH 15% ; S15-1A, STARCH 15% + AMMONIUM SULPHATE 1% ; S15-2A, STARCH 15% + AMMONIUM SULPHATE 2% ; S15-1N, STARCH 15% + SODIUM NITRATE 1% ; S15-2N, STARCH 15% + SODIUM NITRATE 2%]



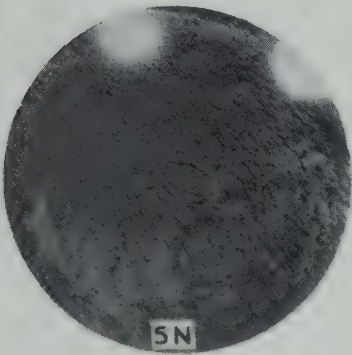
0N



1N

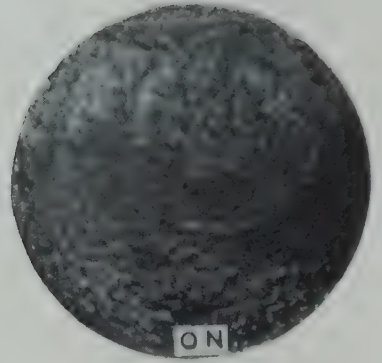


2.5N

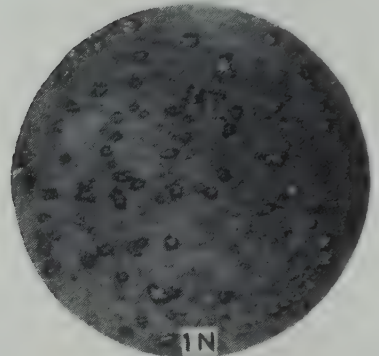


5N

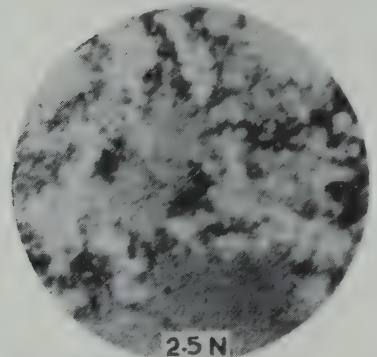
RAILWAY FARM



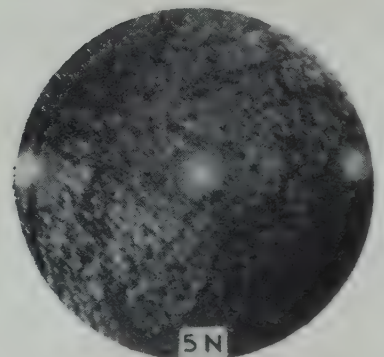
0N



1N



2.5N



5N

NURSERY

FIG. 5.—PLAQUES OF SOIL (FROM KHARAGPUR RAILWAY FARM AND NURSERY) SHOWING THE EFFECTS OF DIFFERENT CONCENTRATIONS OF CZAPEK'S SOLUTIONS (0 N, CONTROL ; 1N, NORMAL CZAPEK'S SOLUTION ; 2.5N, 2.5 NORMAL CZAPEK'S SOLUTION ; 5N, 5 NORMAL CZAPEK'S SOLUTION)



from cultivated and manured plots would be very high. In fact, the number and types of organisms would be numerous and the plates prepared would be crowded with colonies. But the enrichment exerts a selective influence on the growth of organisms. Hence, unless the nutrients were present in relative optimal abundance, a few selected species could grow on the soil surface. It could be noted that general cultural media such as nutrient broth and potato dextrose solution helped numerous types of micro-organisms to grow. The inhibition of one species by the other cannot then be demonstrated as a consequence of overriding of large number of species in a limited space. Demonstration of inhibitory action under these conditions would need isolated growth by dilution of the soil with sterile soil and this seems to be a further line of study.

The enriched soil plaque prepared with starch and ammonium sulphate stimulated the growth of a large number of fungi and bacteria and thus the selective effect of starch was reduced (Fig. 4). But application of sodium nitrate along with starch created an unfavourable physiological environment (Fig. 4) for any micro-organism to grow on the soil surface in most of the soil samples. The physiological antagonism of sodium nitrate to starch probably led to this situation. However, antagonism between a species of *Neocosmospora* and actinomycetes could only be detected in enriched sodium nitrate-soil-starch plaque with saline Gosaba soil.

Czapek's solution, on the other hand, was favourable for the growth of a few species (Fig. 5 and Table 1). At normal concentrations of the ingredients in Czapek's solution growth of *Cunninghamella* on the soil plaques was evident. But higher concentrations of ingredients in Czapek's solution (2.5 times the normal concentration) stimulated the growth of *Verticillium* and *Trichoderma*. At 5 times the concentration of ingredients stimulatory action on the growth of *Penicillia* and *Aspergilli* was conspicuous. This fact has been verified by the authors a number of times by testing a large number of highly manured flower beds and cultivated soils. The soils which proved negative to the growth of *Cunninghamella* with enrichment of Czapek's solution were found to give positive test when inoculated with *Cunninghamella* spores<sup>10</sup>. Isolation of *Cunninghamella*, *Trichoderma* and *Verticillium* from different soil samples can, therefore, be accomplished with much ease, and this might prove an effective tool for the search of specific groups or genera of microbes as sources of antibiotics.

Control of soil-borne plant pathogens by the incorporation of fresh organic matter into soil have been reported by a number of workers<sup>11-13</sup>. The inhibition of phytopathogens in soil was due to the relative increase of the antagonistic soil microbes. Production of antibiotic substances in soil has, therefore, received considerable attention<sup>14-16</sup>. Stevenson and Louchhead<sup>17</sup> have adapted the percolation technique, described by Lees and Quastel<sup>18</sup> and Chase<sup>19</sup>, for the production of antibiotics in soil. Grossbard<sup>15</sup> pointed out that the presence of carbohydrate was the prime requirement for antibiotic production in soil. Our effort, being preliminary in nature, indicates the scope of the enrichment technique for the direct demonstration of antagonism

between micro-organisms on the soil surface. The nutrilites which favour large number and types of organisms in the soil require further studies for their use in soil plaque technique of isolating antagonistic organisms. Though the antagonism between two or more species of micro-organisms could be demonstrated on soil starch plates, yet, the factors which govern this phenomenon have not been well understood. The important finding in this investigation is the selectivity of the nutrilites and Czapek's solution. This facilitates the isolation of *Cunninghamella*, *Trichoderma*, *Verticillium*, *Penicillia* and *Aspergilli* from various soils in which they are present, even when these are associated with a large number and types of other micro-organisms. Species and strains of these genera could be isolated specifically from different soils for studying their antibiotic potentialities.

#### ACKNOWLEDGMENT

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# Isolation of Antibiotic-producing Organisms from Soil Using Dyestuffs

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Study of the effect of dyes on soil micro-organisms shows that fungi are extremely sensitive to the action of Brilliant Green, Congo Red and Crystal Violet to some extent, while actinomycetes were susceptible to Rose Bengal, Brilliant Green and Crystal Violet. However, bacteria were susceptible to Rose Bengal and Crystal Violet and to a lesser degree to others.

The results show that Crystal Violet is a general inhibitor of all the micro-organisms appearing on plates except fungi when acid agar is incorporated with the dye while Congo Red is most useful for the isolation of actinomycetes as growth of fungi is considerably controlled. Both Rose Bengal and Crystal Violet are suitable for the isolation of fungi.

The isolation of micro-organisms on a large scale from soil could be much improved by proper selection of media. Difficulty was often experienced in isolating fungi and actinomycetes from soil because of their low distribution. Dilutions in which abundant number of colonies of the micro-organisms would appear were generally overcrowded with bacterial growth. Certain dyestuffs are well-known bacteriostatic agents and are, therefore, incorporated in the isolation media with a view to reduce the density of bacterial growth on culture plates. Norris' agar (N.A.) medium, Thornton's agar (T.A.) medium and Waksman's acid agar (W.A.) medium were selected as basal media and Rose Bengal, Crystal Violet, Brilliant Green and Congo Red at different concentrations were used as agents to minimize bacterial growth.

## EXPERIMENTAL

Twenty soil samples were plated out for the isolation of fungi and actinomycetes and the soil suspensions were plated out in all the three media with varying concentrations of each of the dyes. The plates were made in quintuplicates and incubated at 25°-30°C. for a period of more than one week. Counts were made and finally the numbers were calculated taking the mean of the counts. The isolation of the growing fungi was made by subculturing on modified C-D agar and that of the actinomycetes on straw infusion broth agar. The production of antibiotic substances was tested by the agar-streak method and the cultures were screened out on the basis of their antibiotic activity.



## RESULTS AND DISCUSSION

The effect of the dyes on soil microflora is shown in Table 1. Rose Bengal was found effective both against bacteria and actinomycetes at concentrations 1 : 5,000 to 1 : 10,000 while fungi were least affected with the above dosage. Brilliant Green, was highly toxic to both fungi and actinomycetes at concen-

TABLE 1—SENSITIVITY OF SOIL MICRO-ORGANISMS AGAINST DYES

DYE MEDIUM		CONCENTRATION OF DYE	TOTAL MICRO-ORGANISMS/G. OF SOIL	BACTERIA Count	FUNGI Count	ACTINOMYCETES Count
Rose Bengal	N.A.	1:1 × 10 <sup>4</sup>	2.9 × 10 <sup>5</sup>	1.0 × 10 <sup>5</sup>	1.6 × 10 <sup>5</sup>	3.0 × 10 <sup>4</sup>
do.		1:5 × 10 <sup>3</sup>	1.3 × 10 <sup>5</sup>	0	1.3 × 10 <sup>5</sup>	0
Rose Bengal	T.A.	1:1 × 10 <sup>4</sup>	3.3 × 10 <sup>5</sup>	2.0 × 10 <sup>5</sup>	1.3 × 10 <sup>5</sup>	0
do.		1:5 × 10 <sup>3</sup>	0	0	0	0
Rose Bengal	W.A.	1:1 × 10 <sup>4</sup>	1.3 × 10 <sup>4</sup>	0	1.3 × 10 <sup>4</sup>	0
do.		1:5 × 10 <sup>3</sup>	9.3 × 10 <sup>3</sup>	0	9.3 × 10 <sup>5</sup>	0
Brilliant Green	N.A.	1:1 × 10 <sup>5</sup>	1.0 × 10 <sup>5</sup>	1.0 × 10 <sup>5</sup>	0	0
do.		1:5 × 10 <sup>4</sup>	3.0 × 10 <sup>4</sup>	3.0 × 10 <sup>4</sup>	0	0
Brilliant Green	T.A.	1:1 × 10 <sup>5</sup>	3.0 × 10 <sup>4</sup>	3.0 × 10 <sup>4</sup>	0	0
do.		1:5 × 10 <sup>4</sup>	0	0	0	0
Brilliant Green	W.A.	1:1 × 10 <sup>6</sup>	4.3 × 10 <sup>5</sup>	4.3 × 10 <sup>5</sup>	0	0
do.		1:5 × 10 <sup>4</sup>	1.3 × 10 <sup>5</sup>	1.3 × 10 <sup>5</sup>	0	0
Congo Red	N.A.	1:5 × 10 <sup>3</sup>	3.6 × 10 <sup>5</sup>	1.3 × 10 <sup>5</sup>	0	2.3 × 10 <sup>3</sup>
do.		1:1 × 10 <sup>3</sup>	4.0 × 10 <sup>5</sup>	3.0 × 10 <sup>5</sup>	0	1.0 × 10 <sup>5</sup>
Congo Red	T.A.	1:5 × 10 <sup>3</sup>	4.0 × 10 <sup>5</sup>	2.5 × 10 <sup>5</sup>	0	1.5 × 10 <sup>3</sup>
do.		1:1 × 10 <sup>3</sup>	5.2 × 10 <sup>5</sup>	1.6 × 10 <sup>5</sup>	0	3.6 × 10 <sup>3</sup>
Congo Red	W.A.	1:5 × 10 <sup>3</sup>	4.3 × 10 <sup>3</sup>	0	4.3 × 10 <sup>3</sup>	0
do.		1:1 × 10 <sup>3</sup>	2.8 × 10 <sup>4</sup>	2.8 × 10 <sup>4</sup>	0	0
Crystal Violet	N.A.	1:5 × 10 <sup>4</sup>	0	0	0	0
do.		1:1 × 10 <sup>4</sup>	0	0	0	0
Crystal Violet	T.A.	1:5 × 10 <sup>5</sup>	0	0	0	0
do.		1:1 × 10 <sup>4</sup>	0	0	0	0
Crystal Violet	W.A.	1:5 × 10 <sup>4</sup>	3.3 × 10 <sup>3</sup>	0	3.3 × 10 <sup>3</sup>	0
do.		1:1 × 10 <sup>4</sup>	2.6 × 10 <sup>3</sup>	0	2.6 × 10 <sup>3</sup>	0
Control	N.A.	0	1.3 × 10 <sup>6</sup>	3.0 × 10 <sup>5</sup>	2.0 × 10 <sup>5</sup>	8.0 × 10 <sup>3</sup>
do.	T.A.	0	10.2 × 10 <sup>5</sup>	2.2 × 10 <sup>4</sup>	2.0 × 10 <sup>5</sup>	6.0 × 10 <sup>3</sup>
do.	W.A.	0	1.0 × 10 <sup>4</sup>	0	1.0 × 10 <sup>4</sup>	0

trations between 1 : 50,000 to 1 : 100,000. The bacterial number was also considerably reduced by the action of the dye. Congo Red was the least toxic and dosages used were between 1 : 100 to 1 : 500. It seemed it was toxic to fungi when used at the above concentrations except at concentration 1 : 500 with acid agar. Crystal Violet is a general inhibitor for all micro-organisms at concentrations between 1 : 10,000 to 1 : 50,000 except when the dye is incorporated with acid agar.

In the foregoing experiments it was found that all the dyes used were more or less inhibitory to micro-organisms in varying degrees. The use of Brilliant Green and Crystal Violet cannot be recommended for the isolation of any of the micro-organisms on the ground of their general toxicity. However, Rose Bengal with Norris' agar may be considered useful for the isolation of fungi at concentrations 1 : 5,000 as both bacteria and actinomycetes are inhibited while Congo Red can be recommended for the isolation of actinomycetes in both Norris' and Thornton's agar with dilutions from 1 : 100 to 1 : 500.

Isolation of *Azotobacter* from soil has been done<sup>1</sup> with Ashby's mannitol medium incorporated with a low dilution of Congo Red. The examination of the soil plates have shown that members of the *Rhizobiaceae*, which are Gram negative, may also develop in such media. Some workers suggested the use of Crystal Violet for the same purpose but the results were not confirmatory.

However, previous observations<sup>2,3</sup> with regard to the use of Rose Bengal for the isolation of fungi were confirmed by the authors. Hence, it can be recommended for selective isolation of fungi. It was experienced that acid agar having pH 3.6 had certain effects on the bacteriostatic or fungistatic action of the dyes. Effect of pH upon the action of the dyes was known to be very pronounced and it was reported earlier<sup>4,5</sup> that the hydrogen ion concentration of the media plays an important role on the bactericidal power of the dyes. The selective action of a dye also depends upon the gram character of the organism. It was observed by several workers<sup>6,7</sup> that Gram-negative organisms are remarkably resistant to the action of Crystal Violet. Similar results<sup>8</sup> were also obtained in the case of Acid-fast organisms although they are known to retain Gram stain.

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# *Volutellospora*: A New Genus of Soil Fungus with Antifungal Activity

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A new genus of soil fungus with interesting morphological characters has been found in Poona soil samples. The name *Volutellospora cinnamomea* Thirum. has been proposed for it. The fungus, on agar plates and surface cultures in liquid media, produces an antifungal substance which while inhibiting the growth of *Myrothecium verrucaria* at higher concentrations, induces remarkable type of morphogenesis at lower concentrations.

During the course of studies on some soil fungi isolated from Poona soil samples, a fungus with interesting morphological characters was observed. The colonies were slow growing, white, with delicate septate mycelium. On most of the agar media employed, such as potato dextrose agar, Czapek-Dox agar, glucose peptone agar and others, the fungus produced abundant fruiting bodies which are of the nature of sporodochia similar to the genus *Volutella* Tode ex Fries. The sporodochia are subsessile to stipitate, developing from a strand of mycelium, cinnamon-yellow, with an expanded discoid apex. There are numerous marginal septate, brown setae which form radiating structures (Fig. 1A). Numerous hyaline, one celled spores are formed which in mature sporodochium are aggregated into a slimy mass.

Following the development of discoid sporodochia of the *Volutella* type, a second type of fruiting body is developed from the same mycelium intermixed with the sporodochia. These are pycnidia which are astomous, dark chestnut-brown in colour and in aggregation appear black in colour. These astomous pycnidia are mostly reniform (Fig. 1B) and are covered by septate brown setae which are similar to those lining the margin of sporodochium. When crushed, the pycnidia open irregularly and extrude out ovate-ellipsoid hyaline spores which are identical with those produced in the sporodochium. In a general way, the pycnidium may be considered as a modification of the sporodochium formed by the upward growth of the margin of the sporodochium to form an astomous pycnidium. The development of two types of fruiting bodies, each quite distinct morphologically from the other, indicates that the fungus represents an undescribed genus closely related to *Volutella*,



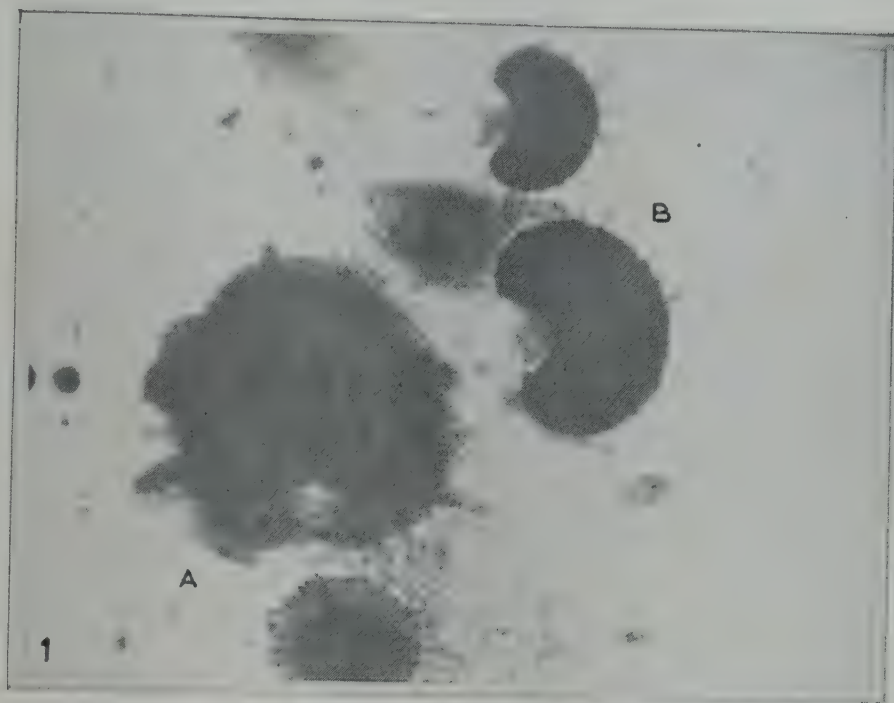


FIG. 1—A: SPOROCHYMIUM OF *Volutella cinnamomea* ;  
B: ASTOMOUS PYCNIDIA ( $\times 250$ )

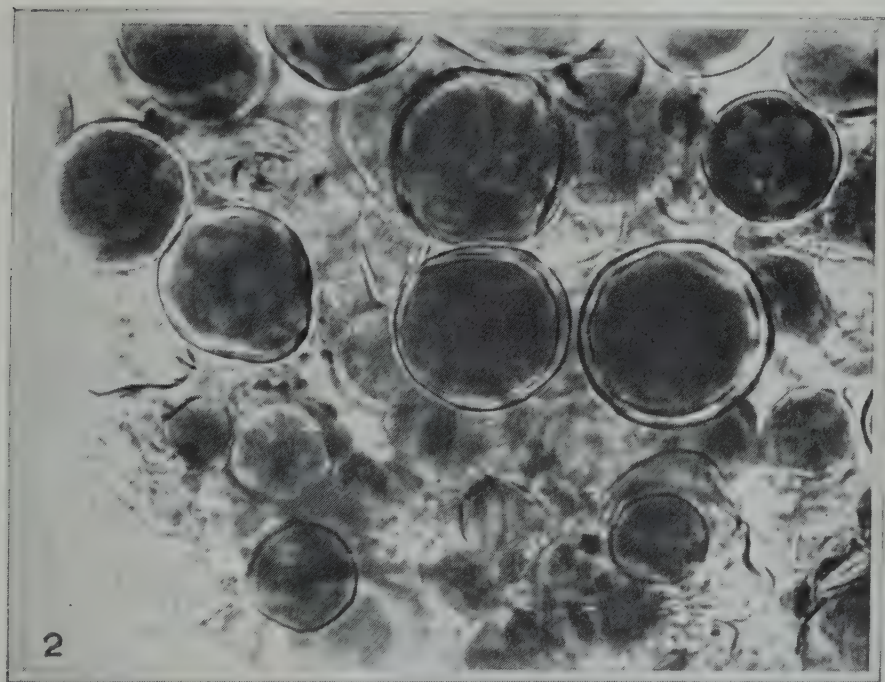


FIG. 2—HYPERTROPHIED CELLS OF *Myrothecium verrucaria* INCITED  
BY THE ANTIFUNGAL SUBSTANCE OF *V. cinnamomea* ( $\times 1800$ )



and the name *Volutellospora* Thirumalachar is proposed for its accommodation. The type species is designated *V. cinnamomea* Thirum.

The antifungal activity of the fungus was first noticed on glucose-peptone agar media in Petri dishes. The fungus inhibited large number of filamentous fungi as was seen in the cross streak tests. Some of the fungi which are sensitive to *V. cinnamomea* are *Penicillium chrysogenum*, *Curvularia lunata*, *Alternaria tenuis*, *Myrothecium verrucaria*, *Fusarium oxysporum* forma *vasinfectum* and others.

Submerged culture of the fungus was tried in various liquid media with different carbon and nitrogen sources. There is only scanty mycelial development which produces abundant spores on short lateral branches. No sporodochia or pycnidia are formed in submerged culture. Due to poor mycelial development in submerged cultures no antifungal activity was observed in the broth filtrates.

In the next series of experiments the fungus was grown on Richard's solution supplemented with 0.1 per cent yeast extract (Difco). The fungus grows slowly and after 25 days' incubation at 24°C. forms a mycelial mat on the liquid surface. Numerous sporodochia are formed giving a pale cinnamon colour. The broth filtrate was tested for antifungal activity using the different test organisms mentioned previously. Table 1 gives the zones of inhibition with the different test organisms when tested with the filtrate by the cup-plate method using 20 ml. of the glucose peptone agar in 10 cm. Petri dishes at pH 6.5.

*Myrothecium verrucaria* was isolated from Poona soil and has been found to be a very useful organism for testing antifungal substances produced by various micro-organisms. In culture it produces abundant mass of spores as gummy crusts. When shaken with water, the spores form a thick suspension without the interference of mycelial fragments. The spores are very small in size, one-celled, and the spore suspension may be easily standardized. When plated on agar and incubated at temperatures between 24 to 30°C. the spores germinate within six hours. Spore suspension stored at room temperature for over a month has shown that over 90 per cent of the spores remain viable. The spore suspension may be stored for more than two months at 5°C.

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TABLE 1—ANTIFUNGAL ACTIVITY OF *V. CINNAMOMEA* CULTURE FILTRATE

	ZONE OF INHIBITION mm.
<i>Penicillium chrysogenum</i>	20
<i>Curvularia lunata</i>	30
<i>Alternaria tenuis</i>	20
<i>Myrothecium verrucaria</i>	35
<i>Fusarium oxysporum</i> f. <i>vasinfectum</i>	20

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When *M. verrucaria* was used as test organism for testing the antifungal activity of *V. cinnamomea*, the zone of inhibition was quite sharp. The fungal colonies along the margin of zone of inhibition on microscopic examination revealed a very abnormal type of growth of mycelium. Following this observation, glucose peptone agar plates were seeded in the centre with *V. cinnamomea* and after 5 days' incubation at room temperature, the spores of *M. verrucaria* were sparsely seeded round the colony of *V. cinnamomea* within a distance of 40 mm. Observations after 3 days showed that most of the spores within the distance of 30 mm. failed to germinate or produced a short germ tube which collapsed completely. Beyond 35 mm. from the central colony of *V. cinnamomea*, the test organism showed a remarkable type of growth (Fig. 2). The spores germinated by short stout germ tubes which formed few irregular branches. The hyphae became closely septate, and each cell swelled up 20 to 50 times the size of the normal hyphal cell. Gradually a mass of spherical hypertrophied cells was formed in aggregation, resembling the undifferentiated type of growth in tumors and galls. Staining reaction with acetocarmine and few other stains showed the occurrence of fragmenting nuclei. While the action of the antifungal substance is comparable with those of carcinogens of known chemical constitution on fungi, none of them so far known possess this type of pronounced action in inciting cell malformation.

When the mass of hypertrophied cells was taken aseptically and replanted in the vicinity of a growing colony of *V. cinnamomea*, it gave rise to further group of undifferentiated mass of cells. When, however, these hypertrophied cells were seeded on fresh agar media in the absence of antifungal substance, the formation of normal germ tubes with long slender hyphae took place similar to the growth of normal colonies. It was, therefore, apparent that the undifferentiated type of growth remained only under the continued presence of the antifungal substance.

# Antifungal Substance from Streptomycete

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A *Streptomyces* species, isolated from Pimpri soil samples, which showed promise of producing an antifungal substance in submerged culture has been examined. Its growth characters on various media have been determined. It resembles helixin and filipin, though different from them. The method followed for extraction and isolation of the active principle is described.

A *Streptomyces* species isolated from soil samples collected at Pimpri, Poona, showed promise of producing an antifungal substance. Vegetative growth in most of the media used was profuse and white and on the advent of spore formation, the colour turned steel blue. Some of the growth characters on various media are as follows.

On Czapek-Dox agar, vegetative mycelium pale yellow in colour, aerial mycelium white, with sparse sporulation after prolonged incubation at 34°C. No diffusible pigment on any of the media tested ; on glucose peptone agar, growth pale cream colour, white aerial mycelium developing rapidly at 34°C. and abundant sporulation after five days ; on Emerson's agar, submerged mycelium spreading, sporulation taking place after long incubation ; on starch-agar, aerial mycelium abundant, with cream coloured submerged mycelium. When tested with iodine, only slight hydrolysis of starch was noticeable. Litmus milk is coagulated and turns blue due to alkaline reaction. Nitrate is reduced slightly to nitrite. Microscopic observations indicated the tenuous branching mycelium and the spores which are formed on sporophores in close spirals.

The antibiotic activity was first tested on agar plates. Several fungal and bacterial organisms were tested, using cross-streak test in 10 cm. Petri dishes with 20 cc. of glucose peptone agar. No antibacterial substance is produced. Table 1 gives the antifungal spectra against some of the test organisms after 4 days' incubation at room temperature, 24°C.

Study of the antifungal substance was made also in submerged culture in 500 cc. flasks with 120 cc. of medium which consisted of soybean meal, sucrose and mineral salts. The broth was tested at different periods for the antifungal substance using *Candida albicans* as test organism.

Comparative studies indicate that the antifungal substance under study is closely related to helixin produced by *Streptomyces* sp.<sup>1</sup> and filipin recently

TABLE 1—ANTIFUNGAL SPECTRA OF SOME TEST ORGANISMS

TEST ORGANISM	ZONE OF INHIBITION mm.
<i>Candida albicans</i>	50
<i>Cryptococcus neoformans</i>	45
<i>Histoplasma</i> sp.	45
<i>Sporotrichum schenkii</i>	40
<i>Curvularia lunata</i>	60
<i>Macrophomina phaseoli</i>	40
<i>Piricularia oryzae</i>	35
<i>Fusarium oxysporum</i> f. <i>vasinfectum</i>	40

described by Ammann *et al.*<sup>2</sup> from *Streptomyces filipinensis*. While helixin has antibacterial property also, filipin is chiefly antifungal affecting both filamentous and yeast type of fungi similar to the antifungal substance (studied by the authors). The general morphological and biochemical characters of *S. filipinensis* and *Streptomyces* under study are also somewhat similar.

#### Extraction and purification of the active substance

A preliminary study was made to develop a suitable method of extraction and isolation of the antifungal principle. The active factor was extractable from the mycelial suspension or filtered broth by amyl acetate, chloroform and ether in the acid pH. The efficiency of extraction was practically the same at pH 2 and pH 6.

From the amyl acetate extract the antifungal substance could be transferred into phosphate buffer at pH 7 and again back into the solvent at acid pH. This method of concentrating the material did not seem to be promising because of unfavourable distribution coefficients. The active principle could, however, be quantitatively absorbed on activated charcoal and eluted with either acetone or 95 per cent ethyl alcohol. The charcoal adsorption technique was adopted to obtain a concentrated preparation which was purified to the extent that a preparation containing dry matter of the order of 10  $\mu$ g. per ml. (corresponding to about 0.1  $\mu$ g. of dry matter in the test plate) gave a measurable zone of inhibition in the assay system.

The following method of extraction and purification was employed: 250 ml. of filtered broth was shaken up vigorously for about 5 min. with 5 g. of active charcoal and filtered under suction. The charcoal containing the adsorbed material was shaken up in 50 ml. of 95 per cent ethyl alcohol and the suspension was poured into a chromatographic tube. The alcohol elute was collected at room temperature and evaporated down to about 25 ml. by heating at 60°C. The purification at this stage was nearly 15-fold compared to the activity in broth. The alcohol extract was further purified by shaking up with an equal



volume of chloroform when an inactive aqueous layer containing appreciable amount of dry matter separated out. The combined alcohol and chloroform extract was evaporated to almost dryness at 60°C. and taken up in a small volume of 95 per cent ethyl alcohol. On keeping the concentrated extract at 5°C. overnight a further crop of inactive material separated out which was removed by centrifugation. Attempts to crystallize the active principle in various organic solvents did not meet with success possibly due to the presence of relatively higher amounts of impurities.

Aqueous solution of the alcohol extract on heating for 10 min. on water bath did not suffer any loss in activity. The antifungal substance thus appeared to be heat stable. The active concentrate in alcohol solution showed an absorption maximum in the neighbourhood of 390 m $\mu$ .

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# A New Antibiotic from *Streptomyces* Species

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**A new antibiotic has been isolated from a *Streptomyces* sp. The antibiotic has been tested by the serial dilution method and found to be active against a number of micro-organisms.**

An antibiotic, apparently hitherto unknown, has been isolated from an actinomycete (*Streptomyces* sp. No. 340). Table 1 gives its cultural characteristics.

**Growth conditions**—The culture was isolated from a local sample of soil by the usual plate dilution method<sup>1</sup>. It was screened by the streak method<sup>2</sup> against six test organisms, viz., *Staphylococcus aureus*, *Escherichia coli*, *Shigella shigae*, *Shigella flexneri*, *Salmonella typhosa*, *Vibrio cholerae*. In view of the promising results obtained during primary screening it was grown in a liquid medium consisting of glucose 1.5, peptone 0.4, meat extract 0.2 (either Lablemco or Bovril) ; sodium chloride 0.5, agar 0.12 per cent, water to volume (final pH of the medium 7.2). The medium was distributed in Roux bottles (200 ml. in each) and sterilized. The bottles were inoculated with spore suspensions prepared from aerial growth on Conn's agar slants. After 7 days' incubation at 28°C. ( $\pm 1^\circ\text{C}.$ ) the bottles were harvested. The final pH was found to be 7.4 (whenever the pH of the culture filtrates was found to be below 7.0 there was a marked depreciation in the antibacterial activity). The activity of the culture filtrate was tested by the cup-plate method against the six test organisms.

Addition of various supplements like corn-steep liquor, yeast extract, distiller's solubles (supplied by the Solan Brewery for trial) alone or in combination did not increase the yield of the antibiotic. Dextrose was found to be the best, optimum concentration being 1.5 per cent and the optima for pH and temperature being 7-8 and 28-29°C. respectively.

## Isolation of the active principle

**From the mycelium**—Mycelium obtained was dried *in vacuo* at 50°C. and ground to a fine powder. It was extracted with chloroform in a Soxhlet for 6-7 days. The chloroform extract was dried over anhydrous sodium sulphate and evaporated to dryness. The dried residue was dissolved in the minimum

quantity of boiling 95 per cent alcohol and the alcohol insoluble fraction (A) removed by filtration. The alcoholic solution was cooled and kept overnight at 4°C. Fine clusters of microscopic orange yellow needles (B) obtained were filtered off and dried. Fraction (A) was found to possess no antibacterial activity while fraction (B), m.p. 333°C. (decomp.) was found to be markedly active against several micro-organisms.

*From the culture filtrate*—The seven-day old active culture filtrate was extracted with ether for several days in the liquid-liquid extractor until the extracted culture filtrate failed to show any antibacterial activity. The ether extract was dried over sodium sulphate and the ether distilled off. From the residue, fractions (A) and (B) were obtained as in the case of the mycelium.

### Antibacterial activity

The antibacterial activity of fraction (B) tested by the serial dilution method is presented in Table 2.

*Physical and chemical properties*—Fraction (B) is insoluble in acetone, water, petrol, ether and benzene. It is soluble in chloroform, ether and sparingly soluble in alcohol. It does not contain any halogen, sulphur or nitrogen. It does not give any colouration with either aqueous or alcoholic solution of ferric chloride.

TABLE 1—CULTURAL CHARACTERISTICS OF AN ANTIBIOTIC FROM *STREPTOMYCES* SP. NO. 340

MEDIUM	10-DAY INCUBATION AT 28°C.
Gelatine	No growth
Glycerol agar (Conn's agar)	White to yellow, thin and spreading Reverse: yellowish Medium: no pigment
Czapek's agar	White, thin and spreading Reverse: white Medium: no pigment
Potato dextrose agar	Creamy white to light grey; raised Reverse: dark brown Medium: pale brown, Starch: hydrolyzed
Glucose agar	White to ash grey, spreading, undulating growth Reverse: creamy white to yellow Medium: no pigment
Calcium malate agar	Dirty brown, thin and spreading Reverse: brown Medium: not coloured
Nutrient agar	Creamy white, shiny Reverse: same Medium: no pigment
Nitrate agar	White turning grey, thin and spreading, powdery Reverse: creamy white Positive nitrite reaction
Tyrosine agar	Thin, creamy white surface growth Reverse: same Medium: no pigment
Milk	Pink ring at the top; slow coagulation and peptonization



TABLE 2—ANTIBACTERIAL SPECTRUM OF FRACTION (B) OF CULTURE NO. 340

TEST ORGANISM	SERIAL DILUTION
<i>Escherichia coli</i>	< 8000
<i>Shigella shigae</i>	< 16000
<i>Shigella flexneri</i>	< 8000
<i>Salmonella typhosa</i>	< 16000
<i>Streptococcus viridans</i>	< 8000
<i>Pseudomonas pyocyaneae</i>	< 8000
<i>Bacillus subtilis</i> B-1-1	> 1500000
<i>B. subtilis</i> 8236	> 2000000
<i>S. aureus</i> (Oxford)	> 800000
<i>S. aureus</i> (Medical College, Lucknow)	> 100000
<i>S. haemolyticus</i>	> 1000000
<i>Candida albicans</i>	< 8000
<i>Corynebacterium diphtheriae</i> in assay broth	> 4000000
in serum broth	No growth
<i>C. diphtheriae</i> (Medical College, Lucknow) in assay broth	> 1000000
in serum broth	> 100000
<i>Mycobacterium tuberculosis</i> H 37 RV	Nil

On acetylation with acetic anhydride and pyridine three different crystalline derivatives, m.p. 205°, 236° and 275°C. respectively were obtained.

From the antibacterial and other properties and melting point it seems that fraction (B) is different from the antibiotics obtained so far from actinomycetes. Further work on characterization of fraction (B) is under way.

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# Antibiotics from the Genus *Fusarium*

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The possibilities of *Fusaria* elaborating compounds of clinical usefulness in antibiotic therapy, particularly in tuberculosis, have been explored. About 130 strains of *Fusaria* representing about 34 species, collected from various parts of the world have been tested for antibiotic production under different sets of culturing conditions and screened against a variety of organisms typical of Gram-positive, Gram-negative and acid-fast groups.

Some strains in twelve species exhibit antibacterial activity, while the crude culture fluids of eleven of these species inhibit the growth of *Mycobacterium tuberculosis*.

The genus *Fusarium*, classed under *Fungi Imperfecti*, has been of interest to biologists from very early times. Because of the widespread occurrence of the genus and the fact that they are causative organisms of wilt in a variety of plants of economic importance, attention has been diverted towards studying the fungus from the point of view of plant pathology and biochemistry.

The discovery that this group of fungi is also capable of elaborating compounds, antibiotic in nature, was first borne out by the investigations of Cook and associates in England. It was shown by Arnstein, Cook and Lacey<sup>1</sup> and Arnstein and Cook<sup>2</sup> that a crystalline blood red pigment isolated from cultures of *Fusarium javanicum* Koorders possessed high degree of inhibitory activity against the acid-fast organism, *Mycobacterium phlei*. Two years later, the same workers isolated from some other species of *Fusaria*, a series of structurally similar antibiotics. Independent of these investigations, Gaumann and associates<sup>3</sup> in Switzerland carried out studies on the antibiotics of some other strains of *Fusaria* and isolated a number of crystalline antibiotics.

These investigations were carried out for exploring the possibility of *Fusaria* elaborating compounds of clinical usefulness in antibiotic therapy, particularly in tuberculosis. It is well known that owing to the great degree of variation in metabolic pathways of micro-organisms, studies relating to antibiotic or pigment production by a given species will have to take into account the nature and types of variations existing among the larger number of strains within the same species. Before any attempt could be made to choose an organism for detailed study of its antibiotic production it becomes necessary to 'screen' or determine the antibiotic spectrum of a relatively large number of species and strains within the same species. About 130 strains of *Fusaria*,

representing about 34 species were collected from various parts of the world, particularly from the U.S.A., Argentina, Holland, Switzerland, England and India and have been tested for antibiotic production under different sets of cultural conditions.

### EXPERIMENTAL

*Fusaria* cultures were maintained by monthly subcultures on a medium of the following composition.

*Potato dextrose malt agar*—Dextrose, 30 g. ; malt extract, 5 g. ; extract of potatoes, 400 g. ; agar agar, 20 g. ; distilled water, 1,000 ml. ; pH, 6.5.

The medium was sterilized at 15-lb. pressure for 20 min. after distributing into test tubes which were slanted while cooling.

### Culture studies in antibiotic production

Since the media employed by different workers for metabolism studies with *Fusaria* including antibiotic and pigment production, have been found to vary in composition (Table 1) which presented a great difficulty in the selection of a medium for routine screening, it was thought desirable to study the relation of antibiotic production to modifications of cultural conditions, so as to evolve a medium that would induce maximum antibiotic production with majority of the strains.

In general two types of variations were made to a Czapek-Dox type of media.

*Nitrogen sources*—A number of nitrogen compounds were used for study of antibiotic activity of the different strains of the fungus, particularly of the influence of the total quantity and type of nitrogen source on antibiotic production. The different nitrogen compounds used were: Sodium nitrite, sodium nitrate, ammonium tartrate, ethylamine hydrochloride, glycine, bacto-tryptone and urea. Antibiotic activity was tested both in the culture fluid and mycelium, starting from the fifth day to twenty-fifth day at intervals of 3 days. It was generally observed that higher amounts of nitrogen sources (0.5-1.0 per cent) were more conducive to antibiotic production than lower

TABLE 1—NITROGEN SOURCES USUALLY USED FOR ANTIBIOTIC AND PIGMENT PRODUCTION FROM *FUSARIA*

MEDIUM	NITROGEN SOURCE	NATURE OF NITROGEN	REFERENCE
Czapek-Dox	Sodium nitrate	Nitrate	Mill & Nord <sup>4</sup>
Rawlin-Thom	Ammonium tartrate	Ammonia	
Richard	Ammonium nitrate	Ammonia & nitrate	Gaumann <i>et al.</i> <sup>3</sup>
Richard	Bacto-tryptone	Amino	Arnstein <i>et al.</i> <sup>1</sup>
Richard	Asparagine	Amino	Cajori <i>et al.</i> <sup>5</sup>



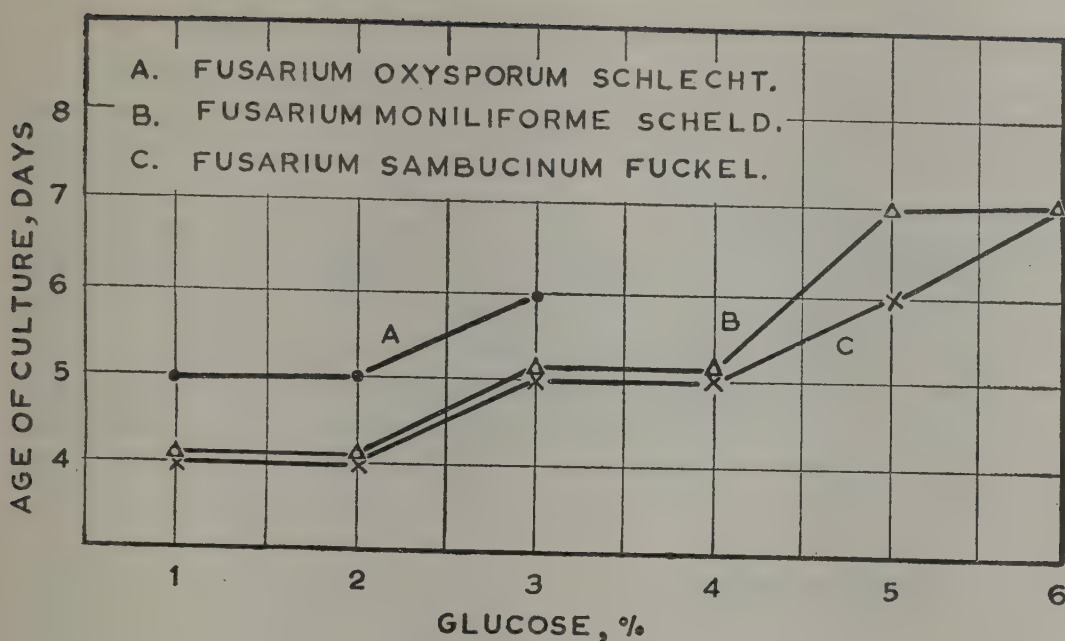


FIG. 1—RELATION OF GLUCOSE TO TIME OF INITIAL APPEARANCE OF ANTI-BIOTIC IN CULTURE FLUID OF SOME *Fusarium* SPECIES

amounts (0.1 to 0.3 per cent). In these investigations the level of glucose was maintained at 2 per cent.

*Carbon-nitrogen ratio*—The importance of C:N ratio has been particularly emphasized by Brown & Horne<sup>6</sup> and Brown<sup>7</sup> in studies relating to pigment production by *Fusaria*. One of the pigments was found to exert antibacterial activity and therefore the effect of C:N ratio on the production of the pigment and also other antibiotics has been investigated. Nitrogen sources were kept at 1 per cent level, and the effect of glucose added at 2 and 5 per cent levels was studied with each type of nitrogen. In all cases, a higher C:N ratio was found to favour pigment production, whether in mycelium or culture fluid.

It was also observed that higher amounts of glucose delay the appearance of antibiotic activity in the culture fluid, although the total yield of the antibiotic was considerably higher with high glucose levels. The effect of glucose levels on the initial appearance of antibiotic in the culture influence of three species of *Fusarium* is presented in Fig. 1.

The results of these studies indicate that variations in the nitrogen source and the C:N ratio not only influence the antibiotic production quantitatively but also appear to modify their composition, as evidenced by differences in antibacterial activity. After much experimentation, the following medium appeared to be satisfactory for routine culture and screening studies.

Nitrogen source, 1 g. ; potassium dihydrogen phosphate, 0.5 g. ; magnesium sulphate, 0.2 g. ; ferrous sulphate, 0.001 g. ; glucose, 2 g. and 5 g. ; distilled water, 100 ml.

The above medium containing low (2 per cent) and high (5 per cent) carbon:nitrogen ratios with bacto-tryptone as the sole source of nitrogen was the final choice for the antibiotic studies.

### Micro-organisms used for antibiotic study

In the initial screening tests five species of bacteria, so chosen as to represent the Gram-positive, Gram-negative and acid-fast groups were used. They were: *Micrococcus pyogenes* var. *aureus* (Gram-positive) ; *Escherichia coli*, *Salmonella typhosa*, *Shigella sonnei*, and *Vibrio cholerae* ogava (Gram-negative) and *Mycobacterium tuberculosis* var. *hominis* H<sub>37</sub>R<sub>v</sub> (acid-fast).

All the bacteria were grown and tested on nutrient broth, except *Shigella sonnei* and *V. cholerae* which were grown on serum broth and peptone water (pH, 8.0) respectively. Modified Youman's medium of the following composition was used for the growth of *M. tuberculosis*:

Asparagine, 5 g. ; potassium dihydrogen phosphate, 5.9 g. ; magnesium citrate, 1.5 g. ; potassium sulphate, 0.5 g. ; glycerine, 20 ml. ; distilled water, 1000 ml. ; pH, 7.2.

### Methods of testing antibiotic activity

Two methods in general, the agar plate or the cup-plate method and the serial dilution method, have been used for the evaluation of the antibiotic activity of the culture fluid and mycelium.

The cup-plate method consisted in punching holes of 8 mm. diam. on agar plates seeded with the organisms to be tested, putting in known volumes of the culture fluids to be tested into the cup and noting the diameters of the zones of inhibition after 24 and 48 hr.

In the serial dilution method, the required concentrations of the antibiotics were prepared in nutrient broth or other liquid media, one drop of 24 hr. culture of the test organism added and the turbidity compared with that of controls after 24 hr. incubation.

### Tests for anti-tubercular activity *in vitro*

Since the results of inhibition obtained on non-pathogenic acid-fast bacteria like *M. phlei* cannot be directly translated to those of pathogenic organisms, it was considered desirable to test the anti-tubercular activity against a virulent strain of *M. tuberculosis*, H<sub>37</sub>R<sub>v</sub> obtained from the National Collection of Type Cultures, England and maintained in this Laboratory on Petrik's medium, the composition of which is as follows:

*Lowenstein's solution*—Asparagine, 0.6 g. ; potassium dihydrogen phosphate, 0.4 g. ; magnesium sulphate (cryst.), 0.04 g. ; distilled water, 12.5 ml.

*Malachite green solution*—2 per cent in distilled water, 3.1 ml.

*Potato extract*—62.5 g. of thinly sliced and freshly peeled potatoes mixed with 250 ml. of 8.4 per cent glycerine in water and autoclaved for 30 min. at 15-lb. pressure, 87.5 ml.

Homogeneous solution of whole egg (125 ml.) and yolk (25 ml.), 150 ml.

From this solid medium the organism was subcultured on to a liquid medium prior to its use for testing the antibiotic activity. Surface culture

technique in varying concentrations of the antibiotic on Youman's medium, read at the end of 3 weeks was the method adopted to evaluate tuberculostatic action as described by Sirsi<sup>8</sup>.

### RESULTS

The results of these antibiotic tests are summarized in Tables 2 and 3. A general report on the species not indicating any activity and the number of strains tested in each is listed in Table 2. Table 3 gives number of strains active in the different species of *Fusaria* and the dilution up to which the antibiotics are active on some of the pathogenic organisms.

The results show that of the 34 species tested, some strains in twelve species elaborate compounds with antibiotic activity.

The complete inhibition of *M. tuberculosis* by the crude culture filtrates of these species of *Fusaria* indicate the wide potentialities of obtaining potent antibiotics from the genus of *Fusaria*.

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TABLE 2—CULTURES OF FUSARIA FOUND INACTIVE

SPECIES	NO. OF STRAINS TESTED
<i>F. albedinis</i> (K. et M.) Malencon	1
<i>F. angustum</i> Sherb.	1
<i>F. bucharicum</i> Jacz.	1
<i>F. caucasicum</i> Let.	1
<i>F. chlamydosporum</i> Wr. et Rkg.	2
<i>F. coeruleum</i> (Lib.) Sacc.	1
<i>F. conglutinans</i> Wr.	2
<i>F. cubense</i> E.F. Sm.	1
<i>F. culmorum</i> (W.) Sacc.	2
<i>F. decemcellulare</i> Brick	3
<i>F. dianthi</i> Prill. et Del.	1
<i>F. graminearum</i> Schwabe	3
<i>F. lini</i> Bolley	5
<i>F. orthoceras</i> App. et Wr.	2
<i>F. perniciosum</i> Hepting	1
<i>F. reticulatum</i> Mont.	1
<i>F. semitectum</i> Berk. et Rav.	6
<i>F. solani</i> (Mart) App. et Wr.	15
<i>F. sporotrichioides</i> Sherb.	1
<i>F. udum</i>	6
<i>Neocosmospora vasinfecta</i>	6

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TABLE 3—ANTIBIOTIC ACTIVITY OF CULTURES OF FUSARIUM SPECIES

SPECIES	No. OF STRAINS TESTED	No. ACTIVE	ACTIVITY AGAINST				
			<i>M. pyogenes</i> var. <i>aureus</i>	<i>E. coli</i>	<i>S. typhosa</i>	<i>V. cholerae</i>	<i>M. tuberculosis</i>
<i>F. avenaceum</i> (Fr.) Sacc.	6	4	+	—	—	—	++
<i>F. dianthi</i> Prill. et Del.	1	—	+	—	—	—	++
<i>F. equiseti</i> (Cda.) Sacc.	1	—	+	—	—	—	—
<i>F. heterosporum</i> Nees.	3	1	++	+	+	++	++
<i>F. javanicum</i> Koorders	3	2	++	—	—	—	++
<i>F. lateritium</i> Nees.	4	1	+	—	—	—	++
<i>F. lycopersici</i> Sacc.	6	4	++	+	+	++	++
<i>F. moniliforme</i> Sheld.	7	5	++	+	+	++	++
<i>F. oxysporum</i> Schlecht.	16	7	++	—	—	—	++
<i>F. sambucinum</i> Fuckel.	5	3	++	—	—	—	++
<i>F. scirpi</i> Lamb. et Ftr.	6	4	++	—	+	—	++
<i>F. vasinfectum</i> Atk.	10	6	++	+	+	++	++

—, no inhibition ; +, 1/10 ; ++, 1/25 and +++ , 1/50 by culture fluid

## ACKNOWLEDGMENT

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# Oxysporin : A New Tuberculostatic Antibiotic from *Fusarium* Species—Cultural Studies on its Production

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Cultural conditions, viz., pH of medium and nature and quantity of carbon and nitrogen sources for the maximum elaboration of oxysporin by *F. oxysporum* Schlecht, strain 549, have been established.

During an initial survey on antibiotic elaboration by the genus *Fusarium*, several antibacterial substances were isolated from the different species. It was observed that a large number of strains of *Fusarium oxysporum* Schlecht, among other species, showed varying levels of activity. Among the many strains tested, strain 549, originally obtained from the *Ministry of Agriculture, Government of the Republic of Argentina*, exhibited maximum antibiotic action.

Subsequent isolation in the crystalline state, and examination of the properties, chemical and biological, of the antibiotic revealed that it did not resemble any of those compounds previously isolated from this species<sup>1,2</sup>. This compound, named oxysporin, has a melting point of 78°C. and an optical rotation (in chloroform) of 80.19°. Oxysporin possesses activity against Gram-positive organisms at concentrations ranging from 1-10 g./ml. and low activity against the Gram-negative group. It exhibits an activity of high level against the virulent human strains of tubercle bacilli, particularly the H<sub>37</sub>R<sub>v</sub> strain, ranging from 0.1 to 1 µg. per ml. This paper concerns itself with studies relating to effect of cultural modifications on the biological synthesis of the antibiotic by *F. oxysporum* Schlecht, strain 549.

## EXPERIMENTAL AND RESULTS

*Effect of nitrogen*—The effect of different nitrogen sources on the metabolism of the organism has been studied. The dry weights of mycelia and total lipid synthesis have been taken as representing indices of growth and metabolism, and antibiotic production, as evidenced by inhibition of a strain of *Micrococcus pyogenes* var. *aureus* both by the cup-plate and serial dilution



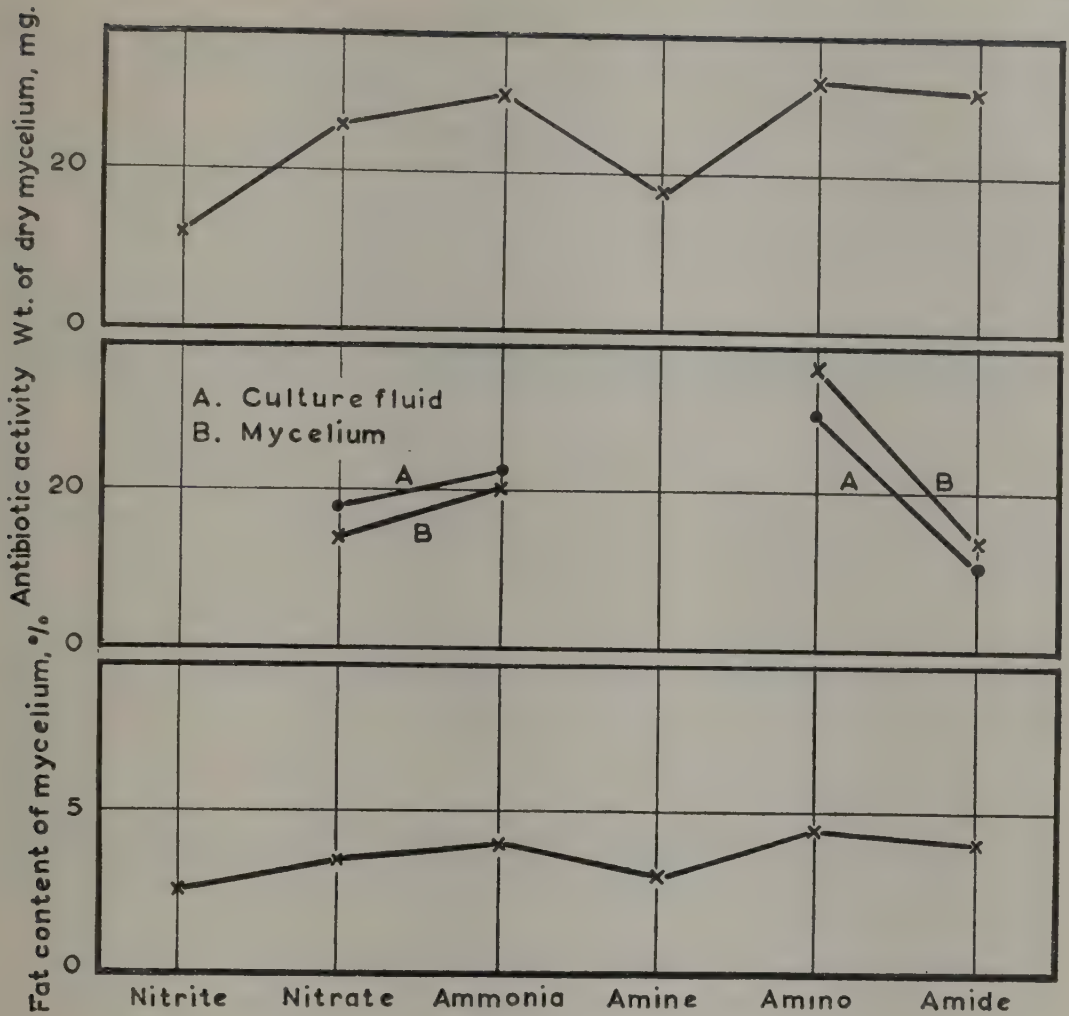


FIG. 1—EFFECT OF NITROGEN SOURCE ON ANTIBIOTIC GROWTH, ACTIVITY AND FAT FORMATION BY *Fusarium oxysporum* SCHLECHT

methods. *Mycobacterium tuberculosis* H<sub>37</sub>R<sub>v</sub> (virulent human strain), has also been used by the serial dilution method, although best expressions of quantitative data on antibiotic synthesis were obtained with the use of *Micrococcus pyogenes* var. *aureus*.

It was observed that, among the many sources of nitrogen compounds tested, nitrite and primary amino-nitrogen compounds inhibited the growth and metabolism of the fungus and there was no detectable antibiotic activity (zone of inhibition of *M. aureus*) either in the culture fluid or in the mycelium. Maximum activity was obtained with amino-nitrogen sources, such as glycine and a complex mixture such as bacto-tryptone. Ammonium salts of organic acids (citrate or tartrate) gave less activity, nitrate-nitrogen still less, with amide-nitrogen (urea or acetamide) the least (Fig. 1). The investigations on the influence of nitrogen sources on antibiotic production were carried out using a simple medium of inorganic salts with dextrose (2 per cent) as the carbon source, and the nitrogen compounds were added in such proportions as would represent a final concentration of 0.5 per cent nitrogen.

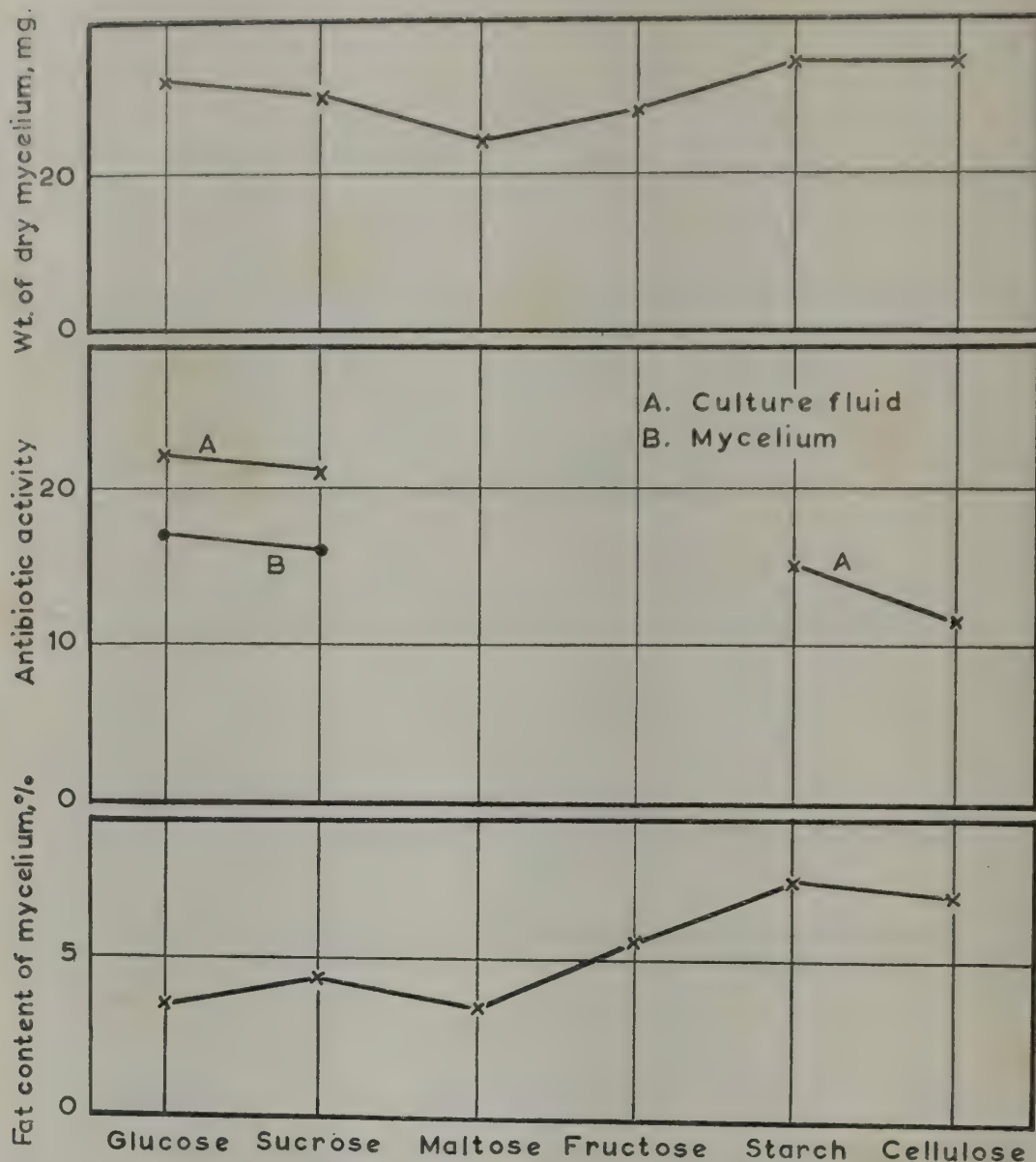


FIG. 2—EFFECT OF CARBOHYDRATES ON ANTIBIOTIC GROWTH, ACTIVITY AND FAT FORMATION BY *Fusarium oxysporum* SCHLECHT

Antibiotic synthesis was followed from the fifth day to the thirtieth day at three-day intervals, and it was observed that the maximum activity was obtained in almost all cases between the fourteenth and twenty-second day of growth.

Having thus established that amino-nitrogen sources elicit maximum production of antibiotic, the effect of various sources of carbon was next tried.

*Effect of carbon*—A number of carbohydrates as sources of carbon have been investigated for the elaboration of antibiotic by the organism. Two aspects have been considered. First, the qualitative effect of these carbon sources and second, the effect of varying the carbon-nitrogen ratio on the relative distribution of the antibiotic between the culture fluid and mycelium.

Using bacto-tryptone as the sole source of nitrogen, the effect of glucose, sucrose, potato starch, cellulose, maltose, fructose and glycerol has been studied.

In these investigations it has been observed that glucose and sucrose are able to stimulate oxysporin production to the maximum, while the others have shown little or negligible activity. These investigations were made using carbon sources at 2 per cent level (Fig. 2).

The relative distribution of the antibiotic between the culture fluid and the mycelium was to a great extent modified by the carbon-nitrogen ratio. While glucose or sucrose of low levels (from 1 to 3 per cent) tend to retain activity largely in the culture fluid, higher carbohydrate levels (from 4 to 7.5 per cent)

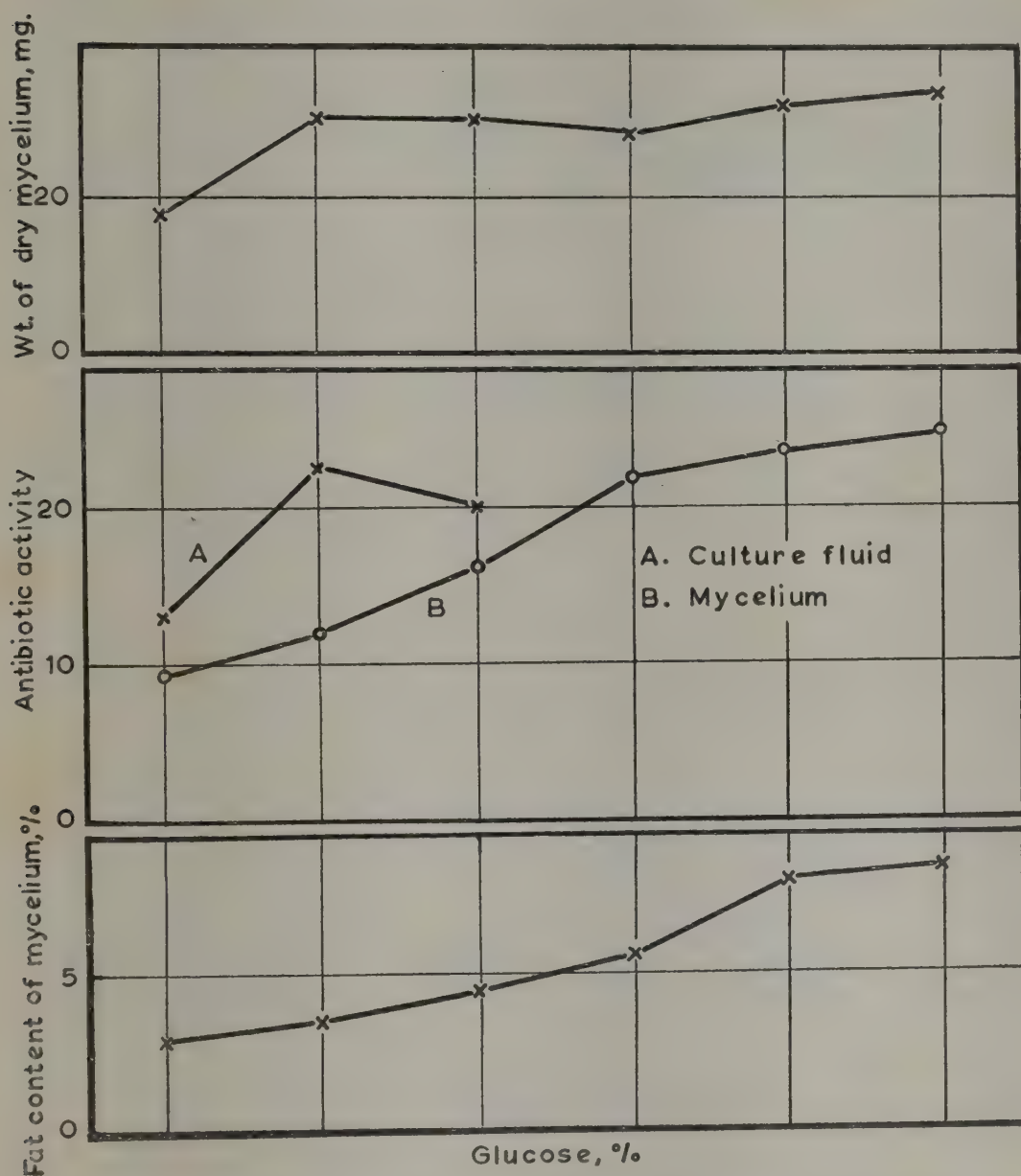


FIG. 3—EFFECT OF GLUCOSE ON THE ANTIBIOTIC GROWTH, ACTIVITY AND FAT FORMATION BY *Fusarium oxysporum* SCHLECHT



show no detectable activity in the culture fluid. All the activity was concentrated in the mycelium, which showed a corresponding increase in total fat (Fig. 3). It would appear that the antibiotic is more soluble in the lipid fraction than in water, or that the compound is not so readily diffusible into the culture fluid in view of the barrier formed due to heavy fatty infiltration within the mycelium. However, it has been observed that the maximum yields of oxysporin have been obtained with glucose levels of 5 per cent or more, and those obtained at lower levels of glucose have been relatively less.

*Effect of initial pH*—Variations in the initial pH of the medium from 4 to 8 considerably affected the rate and total production of oxysporin, and a pH ranging from 5.5 to 7 showed optimal activity. In all these studies growth was induced by surface culture and incubation carried out at 28 to 30°C.

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# Screening of Some Reputed Indigenous Plants for their Anti-tubercular Activity

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*In vitro* studies on the volatile oils from the leaves, roots and flowers of *Adhatoda vasica* Nees, stems of *Leea aequata* Linn., leaves of *Azadirachta indica* A. Juss. and rhizomes of *Acorus calamus* Linn. and *Alpinia galanga* Willd. show that they have marked anti-tubercular activity.

The plants taken up for investigation are especially reputed to be of value in indigenous medicine for the treatment of pulmonary and other forms of tuberculosis. The alcoholic extracts of various parts of the plants were screened against pathogenic test organisms. Later the volatile principles of these plants which showed some antimicrobial activity were tried as it is generally believed by practitioners of indigenous medicine that the anti-tubercular activity of these plants is due to the volatile principles present in them. Of the plants screened so far, the volatile principles of the following five plants were found to possess marked anti-tubercular activity in *in vitro* studies.

## EXPERIMENTAL

Youman's modified medium was used for determining anti-tubercular activity. The tubes containing the drugs in desired concentration were inoculated with various strains of *Mycobacterium tuberculosis* incubated at 37°C. and the results recorded after 7 to 14 days.

For determining the anti-bacterial spectrum, 0.5 ml. of the drug was added in different concentrations to 0.5 ml. of nutrient broth, pH 7.2. The tubes were inoculated with 5 mm. loopfuls of 24 hr. broth cultures of various organisms, inoculated at 37°C. and the results read after 24 hr.

*Extraction of the oils*—The volatile principles were obtained by steam distillation of the various parts of the plants (dried under shade) which are of medicinal value.

## RESULTS

### *Adhatoda vasica* Nees<sup>1</sup>

*Anti-bacterial spectrum*—The oil obtained from the leaves inhibits the growth of 0.01 mg./ml. of B19-4 (Human) strain of *M. tuberculosis* in a concentration

of 2  $\mu\text{g.}/\text{ml.}$  and that of B19-3 (Bovine) strain completely in a concentration of 5  $\mu\text{g.}/\text{ml.}$  and partially in a concentration of 2  $\mu\text{g.}/\text{ml.}$  while the growth of B19-1 (Avian) strain is inhibited in a concentration of 5  $\mu\text{g.}/\text{ml.}$  The growth of the streptomycin resistant strain is inhibited in a concentration of 10  $\mu\text{g.}/\text{ml.}$  The oil from flowers and roots is less effective in inhibiting the growth of various strains of *M. tuberculosis*. It inhibits the growth of *M. tuberculosis* completely in a concentration of 20  $\mu\text{g.}/\text{ml.}$  and partially in a concentration of 10  $\mu\text{g.}/\text{ml.}$  The oil does not inhibit the growth of *Micrococcus pyogenes* var. *aureus*, *Salmonella typhosa* syn. *Bacterium typhosum*, *Escherichia coli*, and *Pasteurella pestis* even in a concentration of 500  $\mu\text{g.}/\text{ml.}$  The action of the oil is specific.

**Toxicity**—The oil when injected subcutaneously in a dose of 2.3 g./kg. body weight does not produce any toxic symptoms.

The oil does not seem to exert toxic action against *Paramecium caudatum*. In a concentration of 200  $\mu\text{g.}/\text{ml.}$  the paramecia are not killed even in one hour.

#### *Leea aequata* Linn.<sup>2</sup> syn. *Leea hirta* Roxb.

**Anti-bacterial spectrum**—The oil obtained from the stems of *L. aequata* Linn. inhibits the growth of *M. tuberculosis* (Human) strain in a concentration of 10  $\mu\text{g.}/\text{ml.}$  while the growth of B19-3 (Bovine) and B19-1 (Avian) strains is inhibited in a concentration of 25  $\mu\text{g.}/\text{ml.}$  The oil also inhibits the growth of *M. pyogenes* var. *aureus* and *P. pestis* in a concentration of 100  $\mu\text{g.}/\text{ml.}$  and 50  $\mu\text{g.}/\text{ml.}$  respectively, while the growth of *E. coli* and *S. typhosa* is not inhibited even in a concentration of 500  $\mu\text{g.}/\text{ml.}$

The oil does not kill *Paramecium caudatum* at a concentration of 500  $\mu\text{g.}/\text{ml.}$  even in one hour and seems to possess very low toxicity for the protoplasm.

#### *Azadirachta indica* A. Juss. syn. *Melia azadirachta* Linn.<sup>3</sup>

**Anti-bacterial spectrum**—The oil from the leaves inhibits the growth of all the three strains of *M. tuberculosis* and *M. pyogenes* var. *aureus* in a concentration of 12.5  $\mu\text{g.}/\text{ml.}$  The growth of *S. typhosa*, *Salmonella paratyphi* and *Vibrio cholerae* Inaba is inhibited in a concentration of 200  $\mu\text{g.}/\text{ml.}$  while that of *Klebsiella pneumoniae* in a concentration of 500  $\mu\text{g.}/\text{ml.}$

#### *Acorus calamus* Linn.<sup>4</sup>

**Anti-bacterial spectrum**—The oil from the rhizomes of *Acorus calamus* Linn. inhibits the growth of H37-RV (Human), B19-1 (Avian) and B19-3 (Bovine) strains of *M. tuberculosis* in a concentration of 10  $\mu\text{g.}/\text{ml.}$  while that of H52-RS (Streptomycin resistant Human) strain in a concentration of 12.5  $\mu\text{g.}/\text{ml.}$  The growth of *Shigella dysenteriae* Shigae, *V. cholerae* Inaba, *Haemophilus pertussis* and *Diplococcus pneumoniae* is inhibited in a concentration of 0.6 mg./ml. whereas the oil does not show any activity against *M. pyogenes* var. *aureus* and *Streptococcus pyogenes* even in a concentration of 1.0 mg./ml.

**Toxicity**—The LD 50 and 19/20 confidence limit as determined by Litchfield and Wilcoxon<sup>4</sup> are 0.0275 (0.0214 to 0.0354) ml./100 g. body weight



of the guinea-pig. The oil when injected in a dose of 0.01 ml./100 g. body weight of the guinea-pig per day for six weeks does not show any toxic symptoms.

### **Alpinia galanga Willd.<sup>5</sup>**

*Anti-bacterial spectrum*—The oil from the rhizomes completely inhibits the growth of H37-RV (Human), H52-RS (Streptomycin resistant Human) and B19-1 (Avian) and partially that of B19-3 (Bovine) strains of *M. tuberculosis* in a concentration of 25 µg./ml. The complete inhibitory concentration for B19-3 (Bovine) strain is 30 µg./ml. The oil inhibits the growth of *S. dysenteriae* Shigae in a concentration of 0.4 mg./ml. of *H. pertussis*, *S. paratyphi*, *D. pneumoniae* and *E. coli* in a concentration of 0.6 mg./ml. and that of *Salmonella schottmuelleri* in a concentration of 0.8 mg./ml. It does not inhibit the growth of *Str. pyogenes* and *M. pyogenes* var. *aureus* in a concentration of 1.0 mg./ml.

*Toxicity*—The LD 50 and 19/20 confidence limit are 0.068 (0.056 to 0.076) ml./100 g. body weight of the guinea-pigs. It does not produce any toxic symptoms when injected in guinea-pig in a dose of 0.02 ml./100 g. body weight per day for six weeks.

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# Guttiferin : A New Plant Antibiotic

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**Guttiferin has been prepared from *Garcinia morella* Desr. Its antibacterial spectrum has been studied. The toxicity and pharmacological properties of the sodium salt of guttiferin are recorded.**

The occurrence of a new antibiotic, guttiferin, in *Garcinia morella* Desr. was inferred spectroscopically and chromatographically by Rao and Gupta<sup>1,2</sup>. Guttiferin has been prepared from the mother liquors left after the removal of the antibiotic, morellin<sup>3</sup>, and the inactive crystalline phenol, moreollin<sup>2</sup>, by extraction with aqueous sodium carbonate. Further purification and the physical and chemical properties of the substance are still under investigation. In view of its property of forming water soluble salts, a preliminary study of its antibacterial spectrum, toxicity and a few pharmacological properties has been made.

## EXPERIMENTAL AND RESULTS

Guttiferin used in these experiments was prepared by extraction of an ethereal solution of morellin mother liquor solids (100 g.) with 5 per cent aqueous sodium carbonate, after a preliminary extraction with 10 per cent sodium bicarbonate solution to remove the more acidic material. Acidification of the sodium carbonate extract gave a product (52 g.) which was thoroughly washed, dried in high vacuum over calcium chloride and used in these experiments.

### Antibacterial activity

The sensitivity of a few micro-organisms to guttiferin, determined by the tube dilution technique, has been expressed as the minimum inhibitory concentration (MIC) in Table 1.

The antibiotic is active only on the several strains of Gram-positive bacteria tested.

### Acute toxicity

Guttiferin was administered to rats intravenously in the form of its sodium salt in physiological saline. In all other cases it was given as 5 per cent solution in olive oil. The controls were similarly treated with equivalent

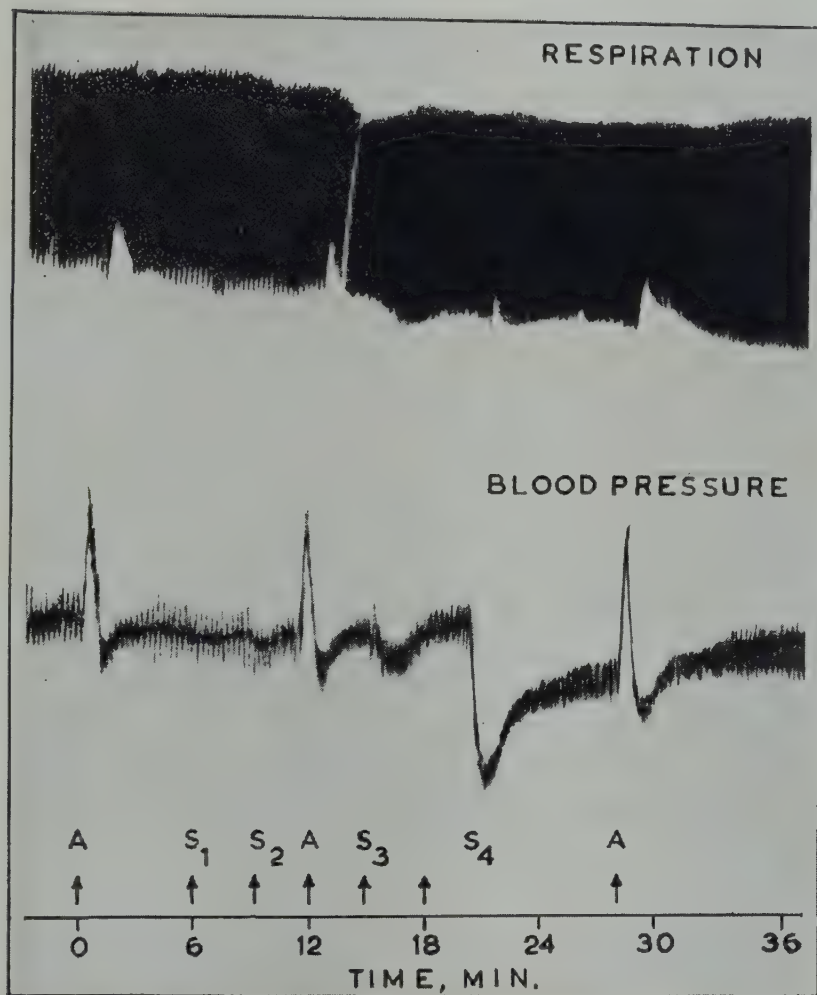


FIG. 1—Kymogram showing the effect of sodium salt of GUTTIFERIN ON BLOOD PRESSURE AND RESPIRATION (DOG WT., 4.1 KG. ; A, ADRENALINE, 3  $\mu$ G. ; DRUG DOSES: S<sub>1</sub>, 5 MG. ; S<sub>2</sub>, 20 MG. ; S<sub>3</sub>, 50 MG. ; S<sub>4</sub>, 100 MG.)



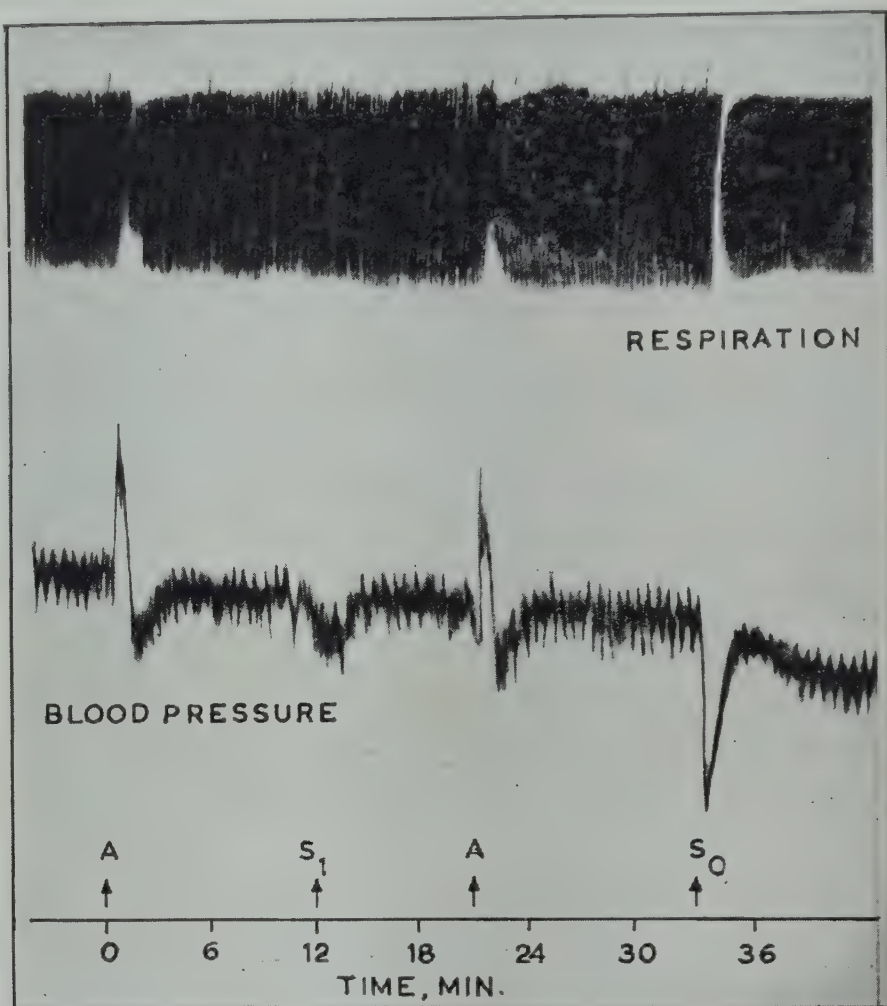


FIG. 2—Kymogram showing the effect of sodium salt of guttiferin on blood pressure and respiration (DOG WT., 4.5 KG.; A, ADRENALINE, 3  $\mu$ g.; DRUG DOSES:  $S_1$ , 100 MG.;  $S_0$ , 150 MG.)

TABLE 1—ANTIMICROBIAL SPECTRUM OF GUTTIFERIN

ORGANISMS*	MIC OF GUTTIFERIN μg./ml.
<i>Micrococcus pyogenes</i> var. <i>aureus</i> , ATCC 9144; AB 58, 59 & 60	0.3-0.4
<i>Bacillus subtilis</i> , ATCC 6633, 9524	0.3-0.4
<i>Streptococcus pyogenes</i> , (β-haemolytic) AB 61	0.4
<i>Escherichia coli</i> var. <i>communis</i> , ATCC 9492 and Kg. <i>Mycobacterium phlei</i> , ATCC 355 <i>Salmonella</i> group, Kg. <i>Shigella dysenteriae</i> , Kg.	>50

\* ATCC: American Type Culture Collection; AB: Antibiotics laboratory Collection; Kg.: Strains from King Institute, Guindy.

amounts of the base without the drug. The general procedure adopted has been described in detail elsewhere<sup>4</sup>.

**Oral toxicity**—No mortality was observed at 1,000 mg./kg. levels.

**Subcutaneous toxicity**—The drug was fairly well tolerated up to 400 mg./kg. single dose levels, although occasionally slight necrosis was observed at higher dosage levels. This manifestation, however, disappeared completely in a few days and the animals then appeared normal. Higher doses could not be administered due to limitations of its solubility in the oil.

**Intraperitoneal toxicity**—It was well tolerated up to 100 mg./kg. levels, but 100 per cent mortality took place at 200 mg./kg. doses within 24 hr. after the administration of the drug. The exact LD<sub>50</sub> in this case was not ascertained.

**Intravenous toxicity**—The sodium salt of guttiferin did not produce any mortality when administered intravenously at levels of 80 mg./kg. However, the LD<sub>50</sub> in this case was estimated to be about 100 mg./kg.

**Toxic manifestations**—The characteristic toxic symptom observed following the administration of large single doses of the drug is paralysis of hind legs coupled with respiratory distress with increased rate before the animal succumbed.

Autopsy and histopathological examination of the surviving animals in above cases showed no evidence of gross toxic manifestations. However, occasionally, a few punctuate haemorrhagic spots around the central vein of the liver were observed.

### Pharmacodynamic effects

The effect on blood pressure and respiration following the intravenous administration of the sodium salt of guttiferin in saline to seconal sodium anaesthetized dogs is shown in the kymograph tracings (Figs. 1 and 2).

Increasing doses from 5 to 50 mg. (Fig. 1) were without effect. Administration of 100 mg. of the substance to the same dog produced a transient fall

in blood pressure returning to the normal level within 5 min. It was not attended by a corresponding change in respiration.

A single intravenous injection of 100 mg. of the drug to a second dog (Fig. 2) caused a very slight fall in blood pressure without any change in respiration. A further dose of 150 mg. produced a sudden drop in blood pressure accompanied by a transient suppression of respiration. The respiratory rate, however, was increased with decrease in the amplitude.

*Effect on smooth muscle*--The effect of the sodium salt of guttiferin on isolated intestine (rat and guinea-pig) and uterus (rat), suspended in oxygenated magnesium-free Tyrode solution at 37°C. is shown in Figs. 3 & 4.

The normal contractions of the nongravid rat uterine horn were not inhibited except for slight augmentation at a concentration of 40  $\mu\text{g.}/\text{ml.}$  of the drug. Addition of 80  $\mu\text{g.}/\text{ml.}$  caused marked inhibition. Acetylcholine given at this stage did not show any spasmogenic activity.

Isolated segments of ileum from rat and guinea-pig were used to study the effect of the antibiotic against the common spasmogenics, acetylcholine, barium chloride and histamine. The contractions elicited by them were not influenced by amounts up to 20  $\mu\text{g.}/\text{ml.}$  However, significant effect was obtained at concentrations of 40  $\mu\text{g.}/\text{ml.}$  or higher.

## DISCUSSION

The antibiotic appears to be fairly specific in its activity towards several strains of Gram-positive bacteria. Little or no activity is exhibited against Gram-negative organisms and mycobacteria. As such it is difficult to envisage a rationale in the use of *Garcinia morella* extracts by practitioners of indigenous medicine in the treatment of enteric affections on this account alone. However, barring the consideration of infective conditions, its activity on isolated intestine in inhibiting the contractions elicited by the common spasmogenics, appear to provide a basis for rationalizing its application as an intestinal spasmolytic. Further work, specially on the properties of the accompanying substances, is obviously necessary to elucidate this aspect.

## SUMMARY

Guttiferin possesses high specific inhibitory action on several test strains of Gram-positive bacteria. It is inactive towards mycobacteria and Gram-negative organisms.

Its sodium salt appears to possess comparatively low toxicity in rats.

When administered intravenously to dogs in fairly high doses, it results in a transient fall in blood pressure accompanied by a little suppression of respiration.

The normal contractions of the nulliparous rat uterus are not inhibited at levels of 20  $\mu\text{g.}/\text{ml.}$  though partial inhibition is produced at a concentration of 40  $\mu\text{g.}/\text{ml.}$  The contractions elicited by the common spasmogenics on isolated intestine are suppressed at concentrations of 40  $\mu\text{g.}/\text{ml.}$  or higher.



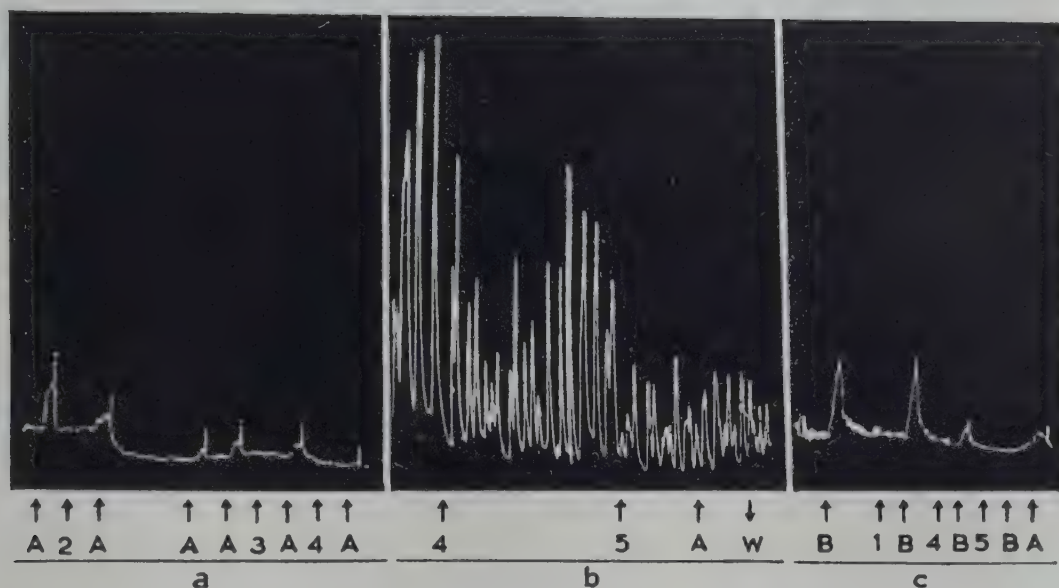


FIG. 3—KYMOTRACED SHOWING THE EFFECT OF SODIUM SALT OF GUTTIFERIN ON ISOLATED RAT INTESTINE (a & c) AND UTERUS (b) (A—ACETYLCHOLINE, 0.05  $\mu$ G. ; B—BARIUM CHLORIDE, 2 MG. ; W—WASH ; DRUG DOSES: 1, 200  $\mu$ G. ; 2, 500  $\mu$ G. ; 3, 750  $\mu$ G. ; 4, 1000  $\mu$ G. ; 5, 2000  $\mu$ G. ; BATH VOLUME, 25 ML.)

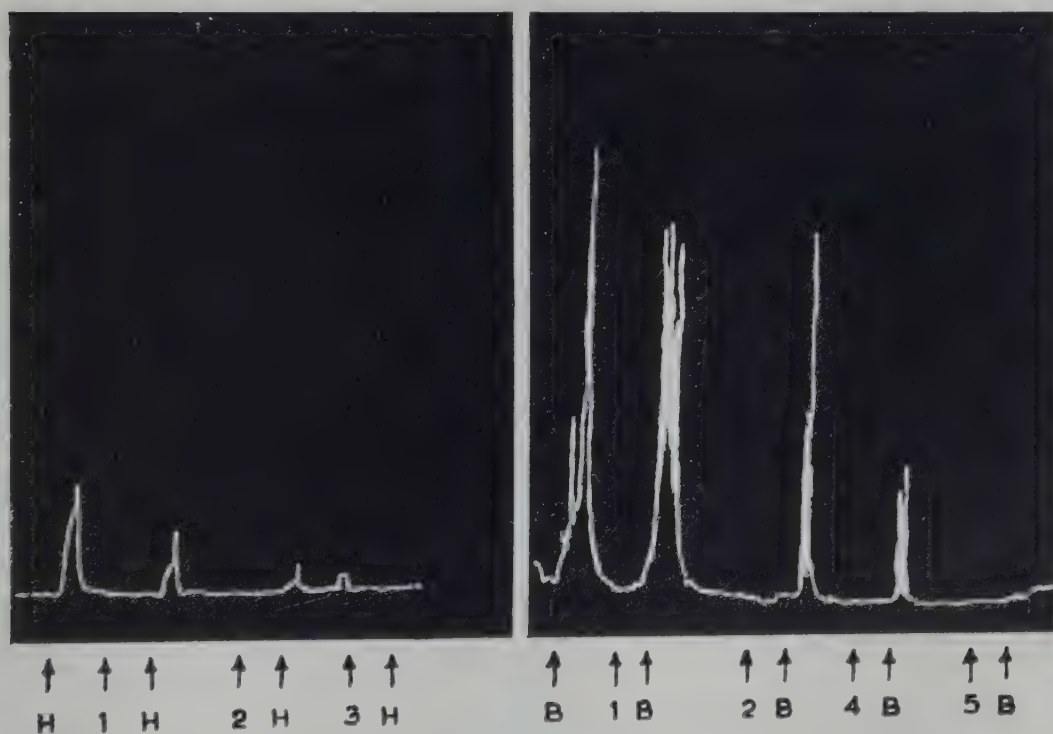


FIG. 4—KYMOTRACED SHOWING THE EFFECT OF SODIUM SALT OF GUTTIFERIN ON ISOLATED GUINEA-PIG INTESTINE (H—HISTAMINE DIHYDROCHLORIDE, 2  $\mu$ G. ; B—BARIUM CHLORIDE, 0.5 MG. ; DRUG DOSES: 1, 500  $\mu$ G. ; 2, 1000  $\mu$ G. ; 3, 1500  $\mu$ G. ; 4, 2000  $\mu$ G. ; 5, 5000  $\mu$ G. ; BATH VOLUME, 25 ML.)



## ACKNOWLEDGMENT

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**Antibiotic Studies on Indian Soil Micro-organisms : Part I—Methods for Screening of Indian Soils for Antibiotic-producing Fungi.** F. M. POONAWALLA, F. FERNANDES & S. S. BHATNAGAR (Bombay).

*J. sci. industr. Res.*, **15C** (1956), 184-85.—A new medium containing dehydrated oxgall and potassium sulphocyanide was developed to supplement the presently available media for the isolation of soil fungi. Although this medium has no advantage over Martin's rose bengal-streptomycin medium as far as the number of fungi developing on plates was considered, the oxgall-KCNS medium being colourless, the identification of soil fungi was easier on this medium than on the Martin's medium. Also, the oxgall-KCNS medium suppressed the growth of other microbial flora equally well, unlike the Martin's medium.

A modified strip test was also developed for primary screening of antibiotic-producing fungi. Its advantage over the existing strip tests is that more than one fungus can be tested against more than one bacterium on each plate (*Abstract*)

**Antibiotic Studies on Indian Soil Micro-organisms : Part II—A Study of Antibiotic Production by Fungi Isolated from Indian Soils.** F. M. POONAWALLA, F. FERNANDES & S. S. BHATNAGAR (Bombay).

*J. sci. industr. Res.*, **15C** (1956), 242-46.—Three hundred and four cultures of fungi were isolated at random from soils from thirty-four locations in India. Of these 30.6 per cent showed antibiotic activity against at least two of the eight test organisms consisting of four Gram-positive and four Gram-negative pathogenic bacteria. The degree of activity ranged from slight to strong, the number of test organisms inhibited by single culture varying from two to eight. In all, 19 different antibiotic spectra were observed. Over 90 per cent of the active fungi belonged to genera *Aspergillus* and *Penicillium*. Two strains of Dermophytes showed marked antibacterial action against all the test bacteria. About 50 per cent of the active fungi were pigment producers. The colour of the pigment being yellow to brown. Freshly manured soils yielded the highest percentage of antibiotic-producing fungi. The results suggest that such fungi are widely distributed and there is a predominance of citrinin- and penicillin-producing fungi in Indian soils. (*Abstract*)

## Some Attempts to Replace Imported Raw Materials in Penicillin Fermentation

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Based on the encouraging results of shake flask experiments, attempts for substituting indigenous materials in place of or in partial substitution of imported raw materials used in penicillin manufacture have proved that the following replacements are possible: Sucrose in place of glucose; groundnut oil as antifoam instead of lard oil and corn oil; partial substitution of lactose with sucrose; replacing about 75 per cent corn-steep liquor by groundnut meal and Sindri calcium carbonate in place of imported precipitated chalk. These new materials are being used on a plant scale at Pimpri.

In finding cheap indigenous substitutes for imported raw materials important consideration must be given to the availability of large quantities of raw materials of uniform quality, their keeping qualities and the type of fermented broth they would yield. Since most of the materials have to be stored for long periods, partially processed products like germinating seed extracts and digested proteinaceous matter of insect origin are of no practical value although shake flask results may be encouraging. Most of the previous workers in India concerned themselves with finding substitutes for corn-steep liquor since it was considered to govern penicillin production to a greater extent than other raw materials. Substitution of the other costly raw materials used in penicillin fermentation, like lactose, glucose, lard oil and corn oil was not given the same amount of attention. Since the commencement of production at Pimpri, experiments were conducted on the use of these raw materials first in shake flasks, and then in the 500-gal. seed vessels used as fermentors, before adopting the process in the large 5,000-gal. fermentors (production tanks). The results of the trials which are of practical significance in penicillin production are presented in this paper.

### Seed-media—substituting sucrose for glucose

In the 500-gal. seed vessels used for germinating spores and developing a vegetative inoculum, 4 per cent glucose is normally employed. The type of nutrition in the seed vessel is quite critical, since a particular type of vigorously growing, mycelial inoculum devoid of much vacuolation is required. It was found first, by experimentation in shake flasks and later, in 500-gal. seed

vessels, that 2 per cent sucrose gave much the same type of inoculum as 4 per cent glucose in the same time. For over a year 2 per cent sucrose is being successfully used in seed media in the commercial production of penicillin.

### Partial replacement of corn-steep liquor by groundnut meal

The use of oilcakes, particularly groundnut meal, as a substitute for corn-steep liquor is in practice in some penicillin plants abroad. The oilcakes are preferred because their composition is much less likely to vary as compared to corn-steep liquor. The problem in this case consists in deciding the right proportion between the groundnut meal and corn-steep liquor. With this end in view, experiments were conducted in shake flasks which indicated that a 3:1 proportion of groundnut meal and corn-steep respectively was the best. The results are recorded in Table 1.

When these experiments were repeated in 500-gal. fermentors, yields of penicillin with groundnut meal alone were much less than with groundnut meal in combination with corn-steep liquor. At present a 3:1 proportion is being used in large fermentors.

### Substituting groundnut oil for lard and corn oil as antifoam

Vegetable oils are generally used in combination with octadecanol as antifoam. These oils also serve as additional carbon sources for the fungus<sup>1</sup>. In the shake flask tests, refined and unrefined groundnut oils were as effective as lard oil or corn oil in antifoam efficiency and did not interfere with yields. Further, lard oil has the disadvantage that it sometimes solidifies in the form of pellets from the medium, becoming ineffective as an antifoam. This is not the case with groundnut oil. Groundnut oil is now being used exclusively in the plant.

### Partial replacement of lactose

Lactose is the costliest of the raw materials used in penicillin production. Several experiments were carried out for its partial and complete substitution

TABLE 1—REPLACEMENT OF CORN-STEEP LIQUOR BY GROUNDNUT MEAL

	pH	PENICILLIN, <i>u</i> /ml.	
		Chemical assay	Bioassay
Groundnut meal (4%)	7.7	1,700	1,700
Groundnut meal (3.75%) + corn-steep liquor (0.25%)	7.7	1,850	1,800
Groundnut meal (3.5%) + corn-steep liquor (0.5%)	7.8	1,800	1,700
Groundnut meal (3%) + corn-steep liquor (1%)	7.8	1,900	1,900



TABLE 2—PENICILLIN YIELD ON REPLACING LACTOSE BY SUCROSE

	YIELD u./ml.
Lactose (3.7%)	1,800
Lactose (2%) + sucrose (1.7%)	1,700
Sucrose (3.7%)—single addition	1,200

by other sugars. Of these, sucrose was found to yield encouraging results, since it was more slowly utilized than glucose and the *pH* of the medium was also more favourable. The results of one of the experiments are presented in Table 2.

It may be interesting to note that in many cases, the results of the shake flask tests do not exactly correlate with those of highly aerated large fermentors. They are only of qualitative significance. Use of sucrose alone in 500-gal. fermentors has given low yields while partial replacement of lactose has given consistently encouraging results. In the 5,000-gal. fermentors, 2.8 per cent lactose and 0.7 per cent sucrose have given up to 1,900 u/ml. in 65 hr. Analysis of the broth samples indicate that the fungus uses sucrose preferentially to lactose and the growth is vigorous. The average rate of production of penicillin per hour is very high, reaching as much as 65 u/ml./hr. in some fermentations, at the peak period.

Other materials have been tried as substitutes for lactose. *Bassia latifolia* flowers were found to give as much as 800 u/ml. in complete replacement of lactose. But this is only of academic interest.

Calcium carbonate, a waste product of the *Sindri Fertilizers & Chemicals*, Sindri has been successfully used in the manufacture of penicillin, in place of the imported precipitated chalk, without deleterious effect on the yield.

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# A Study of Supplements for Fortification of Worts with Special Reference to Production of Antibiotics

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Three by-products of Indian industry—sludges from distilleries, pupal residues from silk filatures and washings of lac factories (all of which are run to waste)—possess supplemental value for use in the production of antibiotics. An estimate of the availability of these by-products has been made and the fortifying efficiency of two of them for antibiotic production has been compared with that of corn-steep liquor.

Industrial fermentations constitute an important group of bio-technical reactions which result in the production of a variety of end-products, solvents, acids, enzymes, vitamins and antibiotics. The efficiency and the overall yield of these reactions has, in recent years, been stepped up to a spectacular extent, thanks to the fundamental advances in general microbiology, to the improvement of strains of organisms and to a systematic elucidation of the nutritional requirements of the organisms.

The basic ingredient or substrate for every type of fermentation is a carbohydrate provided in the form of a polysaccharide like starch or inulin or a simpler sugar like sucrose, maltose or glucose. The dissimilation of this carbohydrate is accomplished in the desired pathway through a series of enzymes, which are first elaborated by the organism. The speed and efficiency with which these enzymatically active proteins are synthesized, are related to the forms of nitrogen and the nature of the vitamins and growth factors provided in the fermenting medium. Table 1 gives a few of the industrially important organisms together with their essential nitrogen and vitamin requirements.

The organisms vary widely with respect to their requirements of nitrogen, vitamins and other unidentified growth factors. *Lactobacilli*, in general, require nitrogen as amino acids and peptides and a number of vitamins of the B-complex and some growth factors yet unidentified but found to be associated with liver extract, yeast extract and malt sprouts. The nutritional requirements of *Aspergillus niger* producing citric acid are relatively simple while the penicillin-producing fungi require the addition of corn-steep liquor

TABLE 1—NUTRITIONAL REQUIREMENTS OF SOME INDUSTRIALLY IMPORTANT MICRO-ORGANISMS

MICRO-ORGANISM	SOURCE OF NITROGEN	VITAMINS	OTHER UN-IDENTIFIED GROWTH FACTORS
<i>Lactobacillus casei</i>	Peptone, essential amino acids	Riboflavin Pyridoxine Biotin Folic acid Pantothenic acid	Liver extract Yeast extract Malt sprouts
<i>Acetobactor sub-oxydans</i>	Ammonium sulphate	<i>p</i> -Amino benzoic acid	
<i>Clostridium aceto-butylicum</i>	Peptones and prolamines	<i>p</i> -Amino benzoic acid	Yeast extract Bran extract
<i>Saccharomyces cerevisiae</i>	Ammonium salts asparagine	Thiamine Pyridoxine Pantothenate Biotin Inositol	
<i>Penicillium chrysogenum</i>	Nitrates		Corn-steep liquor
<i>Streptomyces griseus</i>	Peptones and proteins		Animal stick liquor Corn-steep liquor Fish extract

TABLE 2—BY-PRODUCTS AVAILABLE FOR FERMENTATION

SOURCE	BY-PRODUCT	QUANTITY tons	METHOD OF CALCULATION
Distilleries	Yeast sludge	1,125	3 oz. of yeast per gal. of alcohol distilled. Present output of alcohol, 12 million gal.
Silk filatures	Pupal residue	5,000	20 per cent yield on the present output of 50 million pounds of cocoons
Lac factories	Insect residue	This quantity is being worked out in collaboration with the Indian Lac Research Institute, Ranchi	

TABLE 3—VITAMIN CONTENTS OF RESIDUES ASSAYED MICROBIOLOGICALLY

(μg./g. of moisture-free material)

	YEAST	SILK	LAC INSECT
Thiamine	15	20	14
Riboflavin	50	75	..
Pyridoxin	30	3	162
Niacin	50	270	675
Pantothenic acid	40	250	270
Biotin	2	1.2	2.7
Folic acid	16	..	..
Inositol	..	980	27



TABLE 4—FORTIFYING EFFICIENCY OF BY-PRODUCTS COMPARED WITH CORN-STEEP LIQUOR

(Units of antibiotic/ml. of fermented beer)

	NO SUPPLEMENT	LAC INSECT EXTRACT	SILK PUPAE EXTRACT	CORN-STEEP LIQUOR
Penicillin	9.0	49.0	42.0	49.0
Streptomycin	15.0	40.0	46.0	34.2

which has been found to stimulate the production of the antibiotic. *Streptomyces griseus* which gives streptomycin, requires highly complex forms of nitrogen and supplements in the form of animal stick liquors, fish extracts and other complex natural products of indefinite composition.

Successful fermentations can be carried out only if these essential supplements are present in the fermenting medium in an adequate concentration. Carbohydrates—the starches and the sugars—are derived from grains and plant extracts or often from acid hydrolysates of cellulose. Worts of fermenting mash, prepared from these sources are seriously deficient or imbalanced with respect to the other essential constituents. The medium has, therefore, to be fortified with the supplements which have to be naturally obtained from relatively inexpensive sources. These are to be found among the by-products of agriculture and industry.

A determination of their supplemental or fortification value with respect to type of wort and organism, is an essential requisite for an efficient and rational control of the fermentation. Among the many products which are readily available and easily exploitable are: (a) yeast sludge from distilleries, (b) pupal waste from silk filatures, and (c) wash liquors from seed lac factories<sup>1</sup>. Table 2 gives an idea of the quantities which are, at the moment, available.

The vitamin content of these residues has been microbiologically assayed and the results are presented in Table 3.

Data pertaining to the fortifying efficiency of these by-products with reference to antibiotic production are given in Table 4.

The amino acid analysis of these residues has not yet been carried out and their supplemental value with regard to the production of other fermentation products remains to be determined.

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# Studies on the Supplemental Value of Indian Pulse Extracts in Penicillin Production

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The supplemental value of a few of the extracts from indigenous pulses on the production of penicillin has been investigated. Bengal gram extract has been experimentally shown to be the best of all the extracts that were tried.

Organic supplements have been shown to influence the production of penicillin. Corn-steep liquor<sup>1</sup> increases the yield of penicillin tenfold, i.e., 0.1 per cent. Difco yeast extract exerts a similar effect<sup>2</sup>. Aqueous extracts of freshly ground dried peas constitute a good supplement for obtaining better mould growths and increased yields of the antibiotic<sup>3</sup>.

Two per cent corn oil is known to give an abundant and uniform mould growth and a higher quantity of penicillin<sup>4</sup>.

The present studies were taken up with a view to investigate the value of microbiologically active principles of pulse extracts on penicillin production. Basal medium enriched with graded dosages of the extract calculated on the basis of total nitrogen, was employed and the experimental procedure and the method of assay followed during these studies were similar to those described for penicillin by Abraham *et al*<sup>5</sup>.

## Preparation of extracts of germinated Indian pulses

The pulses which were tried are black gram (*Phaseolus radiatus*), Bengal gram (*Cicer arietinum*) and horse gram (*Dolichos biflorus*). The seeds were steeped in running water for 48 hr. during which period the seeds swell and commence to germinate. After draining, the sprouted seedlings were uniformly distributed in trays furnished with fine wire-mesh bottoms. The trays were then transferred to a cabinet provided with air-holes at the bottom and a flue at the top; the seedlings were allowed to grow in darkness and were periodically sprayed with water. With a view to determine the day on which the maximum amount of asparagine accumulated, the seedlings (200 in number) were sampled out every 24 hr. and immediately blanched in water kept vigorously boiling for this purpose. This treatment served to arrest the enzymatic activity of the tissues. The material was then ground up with water and extracted twice by keeping it on a boiling water-bath for 15 min.

TABLE 1—EFFECT OF PULSE EXTRACTS ON PENICILLIN PRODUCTION  
(Original pH, 6.8 ; test organism, *Staphylococcus aureus*—N.C.T.C. 2153)

		BLACK GRAM EXTRACT		BENGAL GRAM EXTRACT		HORSE GRAM EXTRACT	
		pH after fermen- tation	Area of clearance sq. mm.	pH after fermen- tation	Area of clearance sq. mm.	pH after fermen- tation	Area of clearance sq. mm.
Without extract		4.4	216	4.4	216	4.6	216
With extract corresponding to nitrogen	0.2 mg.	5.0	243	5.0	346	5.8	216
	0.6 mg.	6.6	300	6.0	363	6.8	243
	1.0 mg.	6.6	306	6.6	363	7.0	300

The combined filtrates were acidified to pH 5-6 with acetic acid, kept overnight, filtered and finally made up to 500 ml. The samples were treated in this manner for nine days and the resulting extracts were analysed for total solids and total nitrogen.

The germinated seedling extracts prepared by the above method were added to the original mould growth medium on the basis of the nitrogen content in graded dosages and inoculated with penicillin cultures for antibiotic production. The antibiotic activity was evaluated by adopting the standard cup assay method.

The results are recorded in Table 1.

### DISCUSSION

The results of experiments carried out with extracts of some pulses (Table 1) show that in the case of Bengal gram extract there is a definite and significant increase in antibiotic concentration when the medium is enriched with the extract at 0.2 mg. level. In this case increases as high as nearly 70 per cent are obtained. This is interesting in view of the recent studies of Siddiqui and his collaborators, who have isolated a number of physiologically active substances from Bengal gram. The other extracts, black gram and horse gram, are not so active.

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# Nutritional Factors for Streptomycin Formation

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Investigations on the preparation and composition of wheat bran extract (a medium for streptomycin formation) have been carried out. The studies show that the nutritional factors in the extract influencing antibiotic formation are chiefly the iron content and to a lesser extent the nitrogenous materials probably the basic amino acids. The formation of the antibiotic in supplemented bran extract has also been studied.

Aqueous extract of wheat bran has been reported to be a good medium for streptomycin formation by *Streptomyces griseus*. Experiments were carried out to determine the nutritional factors responsible for the effect. Streptomycin production using the bran extract supplemented with various carbohydrates and minerals was also studied with a view to evolve media for better antibiotic production.

## EXPERIMENTAL AND RESULTS

Wheat bran available in the market was used in these experiments.

The material was dried to constant weight in an air oven at 110°C. and the total solids determined. The dried material was ashed at 800°C. in a muffle furnace.

The total nitrogen in the wheat bran was determined by micro-Kjeldahl method<sup>2</sup>. Non-protein nitrogen was determined in the 15 per cent trichloroacetic acid filtrate. Basic nitrogen was determined in the phosphotungstic acid precipitate. Amino nitrogen was estimated by the Sorensen's method and amide nitrogen by Damodaran's method<sup>3</sup>.

The amount of carbohydrate was estimated by hydrolysing the product with 2 N hydrochloric acid for 4 hr. according to the Hagedorn and Jensen method.

## Preparation of bran extract and its fractionation

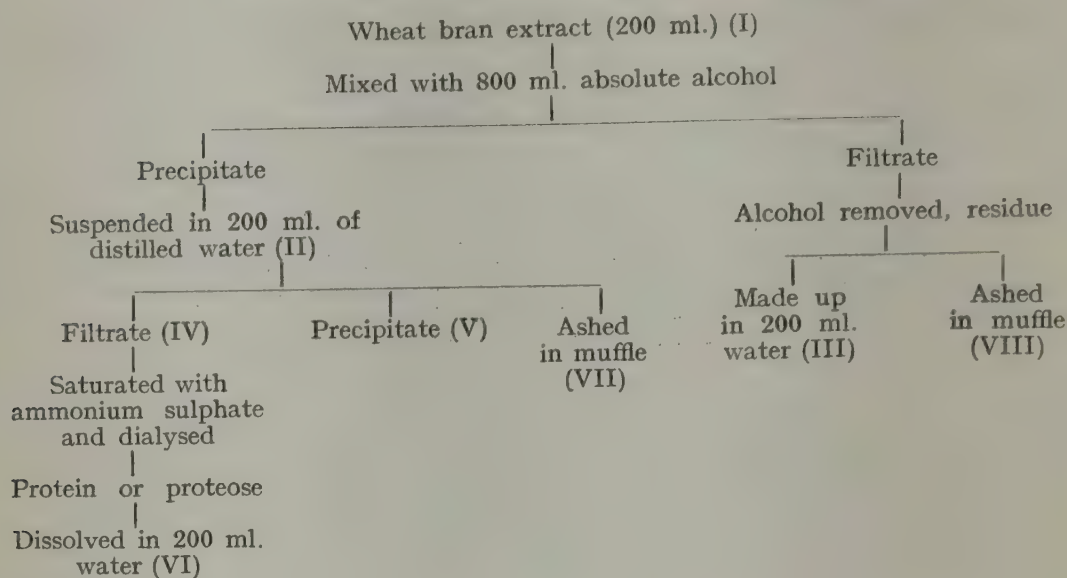
*Bran extract*—Commercial wheat bran (1,000 g.) was mixed thoroughly with water (6,000 ml.). The suspension was aerated (by a water suction pump) for 48 to 60 hr. The suspension was then filtered, distributed in litre conical flasks (about 500 ml. in each flask), plugged and autoclaved for half an hour at 15 lb./sq. in. pressure. The thick white coagulum which settled down during autoclaving was removed by centrifuging. The thick brownish

liquid thus obtained is the bran extract. Its total solids content varied from 3.6 to 4.3 g. per 100 ml. of the extract.

About 30 per cent of the nitrogen was found to be solubilized by the above process.

*Fractionation*—Fractionation of the bran extract was carried out as indicated in Chart 1.

CHART 1—FRACTIONATION OF WHEAT BRAN EXTRACT



### Biological assay

A Waksman's strain of *S. griseus* was used. The assay was carried out by the cup-plate method. For the preparation of the media the total solids of the different fractions were mixed proportionately and made up to volume. In several media thus obtained the pH was about 7. Therefore no attempt was made to adjust it. In the media to which ash was added, no attempt was made to dissolve it.

TABLE 1—STREPTOMYCIN FORMATION IN SUPPLEMENTED BRAN EXTRACT

SUPPLEMENT g./100 ml.	GROWTH mg.	YIELD u./ml.	pH
No supplement	21 (4,5)	110 (6)	7.7 (6)
Sodium chloride, 1.0	..	178 (4)	7.7 (4)
Glucose, 2.0	14 (6)	28 (6)	7.4 (6)
Brown sugar, 2.0	18 (8)	75 (5)	7.3 (5)
KH <sub>2</sub> PO <sub>4</sub> , 1.0 + FeSO <sub>4</sub> & MgSO <sub>4</sub> in traces	15 (6)	85 (4)	7.7 (4)
Glucose, 2.0 + KH <sub>2</sub> PO <sub>4</sub> , 1.0 + FeSO <sub>4</sub> & MgSO <sub>4</sub> in traces	10 (3)	30 (5)	7.4 (5)

Note : Figures in brackets indicate the day of observation

TABLE 2—STREPTOMYCIN FORMATION IN BRAN EXTRACT FRACTIONS

MEDIA No.	BRAN EXTRACT FRACTION	TOTAL SOLIDS <i>mg./100 ml.</i>	ASH OF TOTAL SOLIDS %	NITROGEN <i>mg./100 ml.</i>	STREPTOMYCIN TITRE <i>u/ml.</i>	pH
1	I	2,000	22.6	71.60	114 (7)	7.6 (7.8)
2	II	460	14.2	7.06	15 (6)	7.3 (8)
3	III	1,540	25.2	64.55	13 (6)	7.6 (8)
4	II, III	..	..	..	112 (7)	7.9 (8)
5	II, VIII	460	..	..	78 (7)	7.9 (8)
6	III, VII	1,540	..	..	110 (7)	7.9 (7)

Note : Figures in brackets indicate the day of observation

Some materials which are likely to increase the streptomycin formation were tried with the aqueous bran extract. The results obtained with some of them are given in Table 1. The day-to-day change in the pH of culture fluids was recorded. Growth was determined as the weight of the mycelium dried at 40°C. overnight.

The results obtained by using bran extract fractions are given in Table 2.

#### DISCUSSION

However, the mineral constituents of the bran extract have the most pronounced effect on antibiotic formation. Qualitative analysis of the ash revealed that the ash contained the elements K, Ca, Mg, Al, Na, Fe, Cu, and Mn. Quantitative analysis showed that this ash contains Na, 21.3 ; K, 33.3 ; Ca, 25 and Fe, 0.137 per cent. It was found that the addition of the ash to a basal medium in which there was little streptomycin formation increased the antibiotic titre of culture fluid to about 108 u/ml.<sup>4</sup> A study of the role of the various elements in the ash showed that iron had the most pronounced effect<sup>5</sup>. About 0.7 mg. of iron per 100 ml. of bran extract was found to promote optimum growth of streptomycin. The iron content of bran extract medium was 0.62 mg./100 ml.

The importance of many compounds in the biosynthesis of streptomycin has been recorded<sup>6-9</sup>. Carbohydrate sources are generally accepted to be of little importance in streptomycin formation. The present studies to a great extent support such an assumption. In media rich in glucose the streptomycin titre is very poor. Glucose is known to reduce streptomycin activity. But in glucose containing medium, the maximum pH recorded (7.3 and 7.4) was significantly less than that in media (pH 7.9) in which there was high antibiotic production. Besides, the glucose concentration used in the present investigations was at least twice that generally used in streptomycin fermentation media.

The results clearly show the importance of the mineral constituents. The antibiotic titres in media 5 and 6, both of which contain the entire ash, were



TABLE 3—COMPOSITION OF WHEAT BRAN EXTRACT  
(mg./100 ml.)

Total solids	3,800
Total nitrogen	134.9
Amino nitrogen	15.01
Amide nitrogen	18.5
Protein nitrogen	24.1
Basic nitrogen (phosphotungstic acid precipitable)	81.7
Carbohydrates	475
Ash	860.7

different. This can be ascribed to the difference in the nitrogenous constituents.

An idea regarding the nature of the nitrogenous materials present in the bran extract and its fractions could be had from the analytical data in Table 3.

The presence of large amounts of non-protein nitrogen and basic nitrogen justifies the assumption that significant amounts of basic amino acids are present in the bran extract.

The ethanol soluble fraction (III) is likely to contain the phosphotungstic acid precipitable nitrogen. That streptomycin formation is more in medium 6 than in medium 5 clearly indicates the importance of basic amino acids in streptomycin formation (Table 2). It is possible that preformed amino acids like arginine are preferentially utilized.

The iron content of bran extract increases streptomycin formation. The mechanism is however unknown. The fact that the concentration required for streptomycin formation is more than what is required for growth naturally suggests that the mode of functioning is different.

#### ACKNOWLEDGMENT

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# Studies on Some Physical Properties of Fermentation Broth in Penicillin Manufacture

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The importance of concentration of oxygen in broth on the yield of penicillin in submerged fermentation has been theoretically discussed. The variation in density, viscosity, and surface tension of broth samples of different age from the various fermentors in use in the Pimpri factory has been determined with a view to study the effect of solubility of oxygen and other physical properties on the yield of penicillin. The amount of oxygen in the broth has been found out polarographically. The results obtained in some typical fermentations have been presented.

Production of penicillin is an aerobic fermentation process and the problem of supplying adequate amount of oxygen to the growing mycelium has attracted the attention of workers in this field since 1941 when the *Northern Regional Research Laboratory*<sup>1</sup> commenced the investigations on the submerged fermentation of *Penicillium chrysogenum*. Mycelium derives its requirement of oxygen mainly by diffusion from the broth liquid<sup>2</sup>. There is, however, no evidence to show that it does not absorb the gas directly from the gaseous phase<sup>3</sup>. Rolinson<sup>4</sup> studied the respiration of *Penicillium chrysogenum* and observed that if the concentration of dissolved oxygen was above 0.022 mM/l. at 24°C., the rate of oxygen uptake was independent of the concentration of the solute. The concentration of oxygen in the broth liquid, has therefore to be kept always above this value if oxygen supply is not to be a limiting factor in penicillin synthesis.

In the Hindustan Antibiotics factory, oxygen is supplied to the broth in the fermentors as air bubbled through a sparger located at the bottom of the fermentor. Hixson and Gaden<sup>3</sup> have stated that oxygen encounters two resistances before it enters the bulk of the broth liquid. These are the gaseous and liquid films at the interfaces respectively. If  $p_g$  is the partial pressure of oxygen in the bulk of the gas stream and  $p_l$  the same at the interface, then the force that drives oxygen from the bulk to the liquid film is equal to  $(p_g - p_l)$ . The rate  $R_d$  of mass transfer of oxygen across the interface between the gas and the liquid is given by the mass transfer coefficient  $K_g$  for the gas phase:

$$R_d = K_g \cdot a \cdot (p_g - p_l) \dots \dots \dots (1)$$

where  $a$  is the area of transfer. Similarly if there is no accumulation of

oxygen at any point and  $K_1$  is the mass transfer coefficient in the liquid

$$R_d = K_1 \cdot a \cdot (c_1 - c_l) \dots \dots \dots (2)$$

where  $C_l$  is the concentration of dissolved oxygen at the interface and  $C_1$  that in the bulk of the liquid. In the case of oxygen, the resistance of the gas film may be neglected due to its low solubility. The validity of this assumption is proved by the work of Bartholomew *et al.*<sup>5</sup> who measured the effect of temperature on the absorption of oxygen in sterile fermentation broth. The entire resistance may, therefore, be assumed to exist in the liquid phase and  $R_d$  can be calculated from equation (2). The concentration of dissolved oxygen  $C_1$  can be determined on the basis of Henry's Law as follows:

$$C_1 = H \cdot (p_1) \dots \dots \dots (3)$$

The resistance of the gas film is negligible and since  $P_1 \sim P_g$ , equation (3) can be written as

$$C_1 = H \cdot (p_g) \dots \dots \dots (4)$$

Equation (4) indicates that the concentration of oxygen at the interface is more or less equal to the solubility which can be determined experimentally. In the case of a fermentor,  $a$ , in equations (1) and (2) means the effective interfacial area of the bubbles where transfer occurs.  $K_1$  has a maximum value when the rate of absorption  $R_a$  is maximum.  $R_a$  is solely determined by the rate of formation and degradation of the enzyme complexes which transfer oxygen to the mycelium. When a steady state is reached, the rate of absorption is equal to the rate of transfer and therefore

$$R_d = C_c Q'_{O_2} = K_1 \cdot a \cdot (C_1 - C_l) \dots \dots \dots (5)$$

$$\text{or} \quad K_1 \cdot a = \frac{C_c \cdot Q'_{O_2}}{C_1 - C_l}$$

where  $C_c$  is the cell concentration, mg./ml. in the broth,  $Q'_{O_2}$  is the specific rate of oxygen uptake, mM/(hr.) (g. dry wt.) and  $K_1 \cdot a$  is the absorption rate, mM/(hr.) (litre) (unit concentration difference).

Hixson and Gaden<sup>3</sup> have stated that the following factors mainly effect the absorption rate  $K_1 \cdot a$ . (1) Aeration rate or superficial air velocity; (2) Bubble size or the type of air dispersal device used; (3) Agitator speed and design of agitator; and (4) Physical properties of the liquid phase. In our factory, all the fermentors have more or less the same design and the agitation and aeration are carried out apparently in the same manner. It was thought, therefore, that the variation in the physical properties of the broth may have a profound influence on the yield of penicillin and so also on  $K_1 \cdot a$ .

A detailed investigation has therefore been undertaken to determine the change in the physical properties of the broth liquid with its age. The factors like viscosity, density, surface tension and  $C_l$  and  $C_1$  have been determined and the results obtained in some typical fermentations are presented.

## EXPERIMENTAL

### Method of taking samples

Penicillin fermentation broth is a very complex non-Newtonian fluid, and it is neither a solution nor a suspension which can be studied as such.



*Samples for the estimation of oxygen*—Samples of the fermentation broth were collected in stoppered bottles at various intervals commencing from the time of seeding of the fermentors. The sample bottles were completely filled and thus the contact of air with the sample was eliminated. The broth was then filtered through nylon cloth to remove the mycelium and the filtrate was collected till it completely filled a 25.0 ml. volumetric flask containing deaerated solutions of phenol (5.0 ml. of the saturated solution) and potassium chloride (1.0 ml. of 1M). The time taken for the filtration was 10-15 sec. The flask was then stoppered and shaken to mix the solutions and to kill the micro-organisms which may be present in the filtrate. The solution prepared was used for oxygen analysis.

*Samples for the study of physical properties*—The broth obtained as described above was filtered through Whatman No. 1 filter paper and the filtrate was used for the study of the physical properties.

*Samples for the estimation of non-volatile matter at 100°C.*—The sample of the broth was vigorously stirred and a portion of it was transferred to a weighed bottle. The bottle was heated at 100°C. till it had constant weight. From the loss in weight the percentage of non-volatile matter at 100°C. was calculated.

### Measurement of physical properties

A thermostat with an accuracy of  $\pm 0.05^\circ\text{C}$ . was used for determining the physical properties. Density was measured by the specific gravity bottle method. Ostwald viscometer was used for determining the viscosity. Surface tension was estimated by employing a stalagmometer.

*Strain and composition of the production medium*—A selection from the strain *Penicillium chrysogenum* Wis. 51-20 developed in this factory was used for the fermentations. The production medium contained the following major constituents: Corn-steep liquor, 1.2 ; groundnut meal, 2.25 ; lactose, 3.5 ; calcium carbonate, 0.41 per cent. Groundnut oil with 3 per cent octadecanol was added as an antifoam.

### Estimation of the concentration of oxygen

Several methods are in vogue for the determination of concentration of oxygen in the broth liquid. Cooper, Fernstrom and Miller<sup>6</sup>, and Chain *et al.*<sup>7</sup> utilized the oxidation of sulphite ions to sulphate ions under the catalytic action of copper or cobalt ions as an indicator of the degree of absorption of oxygen ; the oxygen uptake is measured by following the disappearance of sulphite ions. Johnstone<sup>8</sup> found that the reaction is also catalyzed by iron and manganese. The reaction cannot, however, be used in biological media<sup>9</sup> as anticatalysts are present. Wise<sup>10</sup> found that  $K_1$  values calculated by sulphite method and polarographic methods are different. Polarographic methods using a platinum microelectrode or a dropping mercury electrode do not suffer from the difficulties mentioned and therefore, it was chosen in the present work for the estimation of oxygen.

Seven minutes were normally taken to start the oxygen analysis in the

polarograph after taking the sample from the fermentor. For determining the solubility of oxygen in the sample, 10 ml. of the solution as described above was saturated with air by bubbling air through it and then its oxygen content was determined.

The concentration of oxygen was determined by employing a Tinsley polarograph. Oxygen gives two reduction waves at all pH values. Kolthoff and Miller<sup>12</sup> observed that half wave potential of the first wave was independent of the pH of the solution. The diffusion current of the first wave was, therefore, recorded for the estimation of oxygen as done by the previous workers. From the value of the diffusion current obtained, the concentration of oxygen was calculated, on the basis of Ilkovic<sup>13</sup> equation. Potassium chloride solution was added to the broth filtrate to decrease the migration current. The correction for the residual current was obtained by passing purified nitrogen through the solution.

### Accuracy of measurement

The density and viscosity measurements were carried out in the thermostat. The accuracy was better than  $\pm 1.0$  per cent. Surface tension was measured at room temperature which varied by  $\pm 1.5^\circ\text{C}$ . from the temperature reported. The values presented in Tables 1-4 are correct to  $\pm 0.25$  dynes/cm. In the polarographic determination of oxygen and the gravimetric estimation of non-volatile matter, the accuracy is  $\pm 1.0$  per cent.

### RESULTS AND DISCUSSION

The results obtained with a number of batches carried out in the same fermentor (No. 10) indicate that the density, viscosity and non-volatile matter decrease in magnitude with the increase in the age of the broth; surface tension, however, increases (Tables 1 and 2). Studies carried out with other fermentors in this factory have confirmed this variation. The fall in density and viscosity of the filtered broth is to be expected as the material in the production medium which contributes to these properties is gradually used up and metabolised by the mycelium. The gaseous and volatile metabolic products actually escape out with the air and the non-volatile matter in the broth decreases with an increase in the age of the broth. The end-products formed due to chemical actions occurring during fermentation affect the surface tension of the broth considerably and the rise in surface tension with age is observed. Deindoerfer and Gaden<sup>14</sup> have reported the variation of surface tension in a medium having the following composition: Lactose, 4.0; corn-steep liquor, 4.0; sodium nitrate, 0.3; potassium dihydrogen phosphate, 0.05; magnesium sulphate heptahydrate, 0.025; and calcium carbonate 0.4 per cent. Surface tension in this medium first increased from 56 to 60 dynes/cm. at 40 hr. The tension then decreased to 57 dynes/cm. at 60 hr. and again increased to a value of 60 dynes/cm. at 90 hr. The authors have not used any antifoam agent. In this factory, groundnut oil is used as an antifoam after 20 hr. Surface tension, however, increased in all the fermentations studied in contrast to the observations of Deindoerfer and Gaden<sup>14</sup>. They



TABLE 1—VARIATION OF PHYSICAL PROPERTIES OF FERMENTATION BROTH WITH AGE

(Fermentor, 10 ; Batch, 487 ; Date, Jan. 31, 1956)

AGE hr.	DENSITY AT 30°C.	VISCOSITY AT 30°C. cps.	SURFACE TENSION AT 25°C. dynes/cm.	NON- VOLATILE MATTER, (w/w) %	pH	LACTOSE %
0	1.016	1.073	50	5.5	6.5	3.8
5	1.016	1.037	52	5.2	..	..
21	1.015	0.9276	60	5.2	6.35	..
29	1.013	0.9018	63	5.6	6.6	..
45	1.007	0.8708	66	4.8	6.85	1.210
52	1.006	0.8582	67	4.8	6.9	..
71	1.001	0.8435	60	4.5	7.2	0.18

TABLE 2—VARIATION OF PHYSICAL PROPERTIES OF FERMENTATION BROTH WITH AGE

(Fermentor, 10 ; Batch, 517 ; Date, Feb. 15, 1956)

AGE hr.	DENSITY AT 30°C.	VISCOSITY AT 30°C. cps.	SURFACE TENSION AT 24°C. dynes/cm.	NON- VOLATILE MATTER, (w/w) %	pH	LACTOSE %
0	1.016	1.042	50	5.8	6.55	3.61
5	1.016	1.031	52	5.5	..	..
20	1.014	0.9177	60	5.3	6.4	..
28	1.014	0.9051	56	5.0	6.6	..
44	1.009	0.8778	64	4.8	6.8	2.15
52	1.007	0.8658	62	5.0	6.9	1.3
76	1.002	0.8441	64	4.5	..	0.15

have reported that the maximum variation of surface tension is 5-6 per cent from the value at 0.0 hr. The change observed by the authors is nearly 50 per cent. Deindoerfer and Gaden<sup>14</sup> have stated that the viscosity of the filtered broth changed by less than 5 per cent. In the present work, however, the variations were 12 per cent from the mean value. A comparison of the surface tension and viscosity of the filtered broth with that of water indicates that the broth has the properties of a lyophilic sol.

In Tables 3 and 4 the concentrations of oxygen in the broth ( $C_1$ ) is presented. A positive pressure of 8 lb./sq.in. was maintained during fermentation in batch 543. The values of  $C_1$  in this experiment are quite low as compared with the values of  $C_1$ , showing that more oxygen can be dissolved in the broth liquid by efficient aeration. Table 4 gives the analysis of a fermentation with a pressure of 15 lb./sq.in. In general, the values of  $C_1$  are higher than the previous fermentation. Deindoerfer and Gaden<sup>14</sup> have carried out their



TABLE 3—VARIATION OF PHYSICAL PROPERTIES AND OXYGEN CONCENTRATION IN FERMENTATION BROTH WITH AGE

(Fermentor, 10 ; Batch, 543 ; Date, Feb. 28, 1956)

AGE hr.	DENSITY AT 30°C.	VISCOSITY AT 30°C. cps.	SURFACE TENSION AT 25°C. dynes/ cm.	NON- VOLATILE MATTER, (w/w) %	pH	LACTOSE %	OXYGEN, mM/l.	
							C <sub>1</sub>	C <sub>2</sub>
0	1.016	1.050	48	5.4	..	3.61	0.07829	0.2221
4	1.016	1.018	48	5.3	..	..	0.1644	0.2595
20	1.014	0.9173	61	5.2	..	..	0.05595	0.2741
28	1.014	0.9001	66	4.9	6.7	..	0.07709	0.3119
44	1.008	0.8768	67	4.8	7.0	1.56	0.07461	0.3119
52	1.006	0.8728	69	4.9	7.0	0.936	0.1020	0.2562
73	1.001	0.8435	70	5.0	7.0	..	..	..

TABLE 4—VARIATION OF PHYSICAL PROPERTIES AND OXYGEN CONCENTRATION IN FERMENTATION BROTH WITH AGE

(Fermentor, 10 ; Batch, 575 ; Date, Feb. 15, 1956)

AGE hr.	DENSITY AT 25°C.	VISCOSITY AT 35°C. cps.	SURFACE TENSION AT 30°C. dynes/ cm.	NON- VOLATILE MATTER, (w/w) %	pH	LACTOSE %	OXYGEN, mM/l.	
							C <sub>1</sub>	C <sub>2</sub>
4	1.014	0.9166	47	5.3	..	3.60	0.1450	0.2458
20	1.013	0.8266	63	4.8	6.7	..	0.1450	0.2694
28	1.009	0.8021	67	4.7	6.7	..	0.1843	0.2882
44	1.002	0.7743	68	4.6	7.0	0.90	0.1181	0.3260
52	1.000	0.7702	68	4.4	7.1	0.32	0.01891	0.2882
67½	1.000	0.7647	66	4.0	7.3	..	..	..

experiments on a 5-litre fermentor in which a medium which is slightly different from the production medium was used. The observed difference in the variation of the physical properties with the age of the filtered broth described above may depend on the nature of the medium or on the nature of the fermentation that takes place. Calam, Driver and Bowers<sup>15</sup> point out that each particular type of mould, each type of fermentation apparatus and each medium have a characteristic influence on the yield of penicillin.

Previous workers who have reported investigations similar to those presented in this paper, have, however, experimented only with shake flasks and laboratory fermentors and the medium and the strains used by them are different. The work described, in this paper, has been carried out with the big fermentors used in this factory for the production of penicillin. As stated above, the conditions obtained in shake flasks and in laboratory fermentors are very different from those prevailing in the fermentors used for production and this explains some difference in results obtained. A study of the ambient conditions in the fermentors is planned.

## ACKNOWLEDGMENT

The authors are grateful to Dr. K. Ganapathi for suggesting the problem and for his keen interest in the progress of this work. The co-operation given by the fermentation section is acknowledged with thanks.

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## Discussion

DR. K. S. G. DOSS: The work is of high potentialities. The work on oxygen concentration would help in finding out conditions under which lactose consumption can be economized and may contribute to the economy of production.

It appears that the surface tensions of the systems, such as the one the authors are dealing with, show variation with time. This phenomenon deserves attention in interpreting the results.

DR. H. GHOSH: For a particular medium, leaving aside other factors, a good yield of penicillin depends on the rate of aeration as well as on the rate of stirring the particular medium, the latter especially influencing the distribution of air throughout the bulk of the medium. So for each kind of medium these factors should be determined in order to find out conditions of best yields.

DR. V. SUBRAHMANYAN: The physico-chemical data help to punctuate the rate of production of penicillin. Of particular interest is the pH which shifts to the alkaline side. The highest yield of penicillin is noted when pH is 7.3. It should be worthwhile verifying the point.

# Production of Penicillin Using Oilcake Medium

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The possibilities and economics of use of vegetable oilcakes, such as those of mustard, groundnut, sesame, linseed, cottonseed, etc., either singly or in their various combinations as substitutes for corn-steep liquor in penicillin production have been discussed. The advantages of such use of oilcakes, and other factors responsible for the economic production of penicillin have been outlined.

To produce penicillin economically, the following points are to be taken into consideration: (i) cost of raw materials for the medium, (ii) yield of penicillin in the fermentation medium, (iii) strain of penicillin, (iv) loss during extraction and (v) cost of establishment.

## Cost of raw materials

The principal ingredient of the standard medium is corn-steep liquor. As India is lacking in this material, it has to be imported. With imported corn-steep liquor, the medium cannot be cheap enough to compete with the foreign finished product. It is supposed that the quality of corn-steep liquor depends principally upon a certain limit of lactic acid fermentation and also conversion of available nitrogenous material of the washed spent of maize to amino acids. The fermentation cannot be standardized in each batch of corn-steep liquor, as it is always influenced by seasonal variation of temperature and bacterial flora of the atmosphere.

The question might now arise whether the spent wash of corn in starch industry in India can be utilized for the above purpose. The temperature and humidity in India is much more variable than in America. So, it will be more difficult to get a standard grade of corn-steep liquor at a low cost. Even if it can be secured to some extent, there is every possibility of variation of yield of penicillin. It has been noticed that even with the imported corn-steep liquor, the yield varied widely. Another difficulty is that this material does not keep well in tropical climate. It gets fermented and becomes unsuitable for use. The possibility of avoiding fermentation of the corn-steep liquor in cold storage is remote, because during transit by train in tropical



heat from site of the Indian starch industry or during the time that elapses between clearance and transport from the port when it is imported, there is every chance of its being fermented.

After long search for a cheap suitable substitute for corn-steep liquor, we found that vegetable oilcakes such as those of mustard, groundnut, sesame, linseed, cottonseed, etc. individually or their various combinations, make an ideal substitute for corn-steep liquor. These oilcakes are not only abundantly available in India, but their price is also very low in comparison with the imported corn-steep liquor. No special arrangements are required for storing the oilcakes. But the most important advantage is that there is very little variation in the maximum yield of penicillin in oilcake medium as the constituents of the oilcakes do not vary appreciably from lot to lot. Usually about 6 per cent corn-steep liquor is used for fermentation, whereas only  $2\frac{1}{2}$  per cent of oilcake is quite sufficient to give up to 2,400 units per ml. The cost of imported corn-steep liquor comes to about 6 annas per lb. whereas the oilcake costs only  $1\frac{1}{2}$  annas per lb.

### **Yield of penicillin after fermentation**

During the last 4 years the unit yield of penicillin in the fermentation medium containing corn-steep liquor has been considerably augmented by the use of high yielding mutant strains. Most of the manufacturers in foreign countries are obtaining maximum yield up to 3,000 units per ml., the average being about 2,000 units per ml.

The recent drop in price of penicillin is the result of its high yield. This is no doubt due to the great advance made in finding high yielding mutant strains by the use of X-rays or ultra-violet rays. The proper method of conservation of high yielding strain and the isolation of high yielding colonies from the degenerating or diseased colonies of the strain are very important factors in getting high yield of penicillin. The most important advantage of the oilcake medium is that the variation in the maximum yield is very rare, the average yield being 2,200 units per ml. Another advantage of the oilcake medium is that foam formation is minimum and very little antifoam mixture is necessary. Besides, the oilcake medium does not allow pH to rise above 7.6. The reaction during the whole fermentation period remains between pH 7.2 and 7.6, the most ideal pH for penicillin production. The trace of oil in the medium forms a coating inside the mild steel tank and this coating prevents rusting of the tank and thus extends its durability. Also, use of oilcake medium results in almost 98 per cent of the total penicillin in the form of 'G' Penicillin.

The presence of some solid particles in oilcake medium in conjunction with mycelia makes it very suitable for filtration. This not only helps in obtaining a clear filtrate, but also reduces the loss in filtration. Besides, the residue obtained after filtration constitutes a good nutritive alimentation for pigs and poultry.

Though oilcake medium contains oil and their degradation products, the purification and crystallization of penicillin are not attended with any difficulty.

### Cost of establishment

It is quite obvious that if there is a separate organization solely meant for the production and sale of one item such as penicillin, the cost under labour and overhead will be high. After meeting labour cost, overhead and maintenance, the cost of producing penicillin in the country works out to be higher than that of imported penicillin. In other countries the antibiotic industry derives profit from the sale of one or more of other antibiotics at a higher price. When the price of penicillin was high and the margin of profit also fairly high, most of the antibiotic manufacturers have been able to amortise almost their entire capital. A well-organized pharmaceutical concern can utilize its existing scientific personnel, maintenance staff and sales organization for production and sale of penicillin without making any extra expenditure. Except the expenses that are to be made for the purchase of special machines, no capital expenditure for land, building, water connection, etc. which are already existing in such a concern, is required. This will materially lower down the cost of production. Consequently, if antibiotic manufacture is undertaken by an already existing pharmaceutical concern of standing, equipped with workshop, maintenance staff and analytical laboratory, it will always be more economical than an antibiotic unit only for the production of one antibiotic. Besides, there is risk of a particular antibiotic becoming obsolete.

It has been observed that due to the higher price of corn-steep liquor and its higher consumption (6 per cent), the cost of raw materials for one mega unit of penicillin is 6 pies more than when mustard cake is used. So in an output of 10,000 mega units the saving when oilcake medium is used will be Rs. 312-8-0.

### Discussion

DR. V. SUBRAHMANYAN: Can the author indicate the composition of the oilcake mixture?

DR. A. GUHA THAKURTA: The composition of the medium is as follows: Mustard cake, 2.0 ; sesame oilcake, 0.5 ; lactose, 3 ; glucose, 0.5 ; sodium nitrate, 0.3 ; magnesium sulphate, 0.0125 ; potassium dihydrogen phosphate, 0.05 ; and calcium carbonate, 0.5 per cent and tap water.

DR. V. SUBRAHMANYAN: The critical point at issue is whether we can successfully replace corn-steep liquor and groundnut cake with a mixture of mustard oilcake and sesame oilcake as reported by Dr. Ghosh and his colleagues. If that can be confirmed at Pimpri, the results would constitute something really valuable to penicillin manufacture and lead to a considerable saving in cost.

DR. K. GANAPATHI: The mixture of mustard cake and sesame cake has not been tried in this factory, but it will be tried.



# Oilcakes for the Manufacture of Penicillin

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Investigations were undertaken for assessing the suitability of some oilcakes (like groundnut, castor and mahua) for replacing imported corn-steep liquor in the production of penicillin. Groundnut meal has been found to be the best substitute.

The successful production of penicillin is dependent upon: (i) use of strains capable of producing high titres, and (ii) use of appropriate media giving high titres under optimum conditions of fermentation. The present investigation deals with the development of suitable media, using indigenous material. Several materials have been tried but the most significant results were obtained by Moyer with corn-steep liquor—a by-product of the starch industry—which when incorporated in the medium for production of penicillin gave rise to manifold increase in the yields. The production medium in most of the factories consists generally of lactose (carbohydrate), corn-steep liquor, nitrogen and carbon sources, and calcium carbonate for the submerged fermentation processes.

Several workers have tried to find out the actual nature of the substance or substances present in corn-steep liquor responsible for good penicillin yields. With this object in view, corn-steep liquor has been very thoroughly investigated and complete analyses for its amino acids, vitamins, ash, etc. have been carried out. But all these results show the great variability of corn-steep liquor from factory to factory, country to country and even batch to batch in the same factory. Bowden and Peterson<sup>1</sup> suggested the use of sprouted grain for the preparation of active corn-steep liquor. In spite of such an exhaustive analysis of the constituents of corn-steep liquor, it cannot be said that it is exceptional in the production of penicillin.

On account of the variability of the composition of corn-steep liquor, various natural products of indigenous origin were tried in place of corn-steep liquor, e.g., casein hydrolysate, hydrolysed corn, wheat or soya bean, but they did not prove useful. On the contrary, some oilcakes were found equally or even more useful than corn-steep liquor itself under the same conditions (Table 1).

India is the largest producer of vegetable oilseeds in the world. About 30 lakh tons of groundnut are produced annually and about 5-6 lakh tons



of groundnut cake are available, out of which about 2 lakh tons are exported annually. Large quantities of oilcake from cottonseed, coconut, linseed, etc., and cakes from castor seeds and from the seeds of *Bassia latifolia* (mahua) are also available. The cakes from castor and mahua are not useful as animal fodder and are mainly used as fertilizers. Moreover, large quantities of corn-steep liquor are imported. These investigations were undertaken to find the suitability of these indigenous materials as protein source in the manufacture of penicillin.

Analysis of the constituents of the cakes has been made. Some of the results obtained with groundnut, castor and mahua cakes are recorded in Table 2. Analysis of cottonseed cake is in progress.

The proteins have been isolated from groundnut, castor and mahua cakes. The hydrolysed proteins of groundnut meal have been examined by paper chromatography; there appear to be at least fifteen to sixteen amino acids of which cystine, lysine, serine, glutamic acid, proline, tyrosine, and leucine seem to have been characterized.

Shake-flask experiments have been carried out with 125 ml. of the medium in 500-ml. wide mouthed Erlenmeyer flasks. The medium was sterilized with steam at 15 lb./sq. in. pressure for 20 min. These were inoculated with 1 ml. suspension of 20 million spores of the strain, *Penicillium chrysogenum* 5120/H.A. (Hindustan Antibiotics isolate by Dr. M. J. Thirumalachar) and

TABLE 1—USE OF CORN-STEEP LIQUOR AND SUBSTITUTES IN PENICILLIN PRODUCTION<sup>2</sup>

ADDITIVE CONSTITUENT g./l.	MAXIMUM PENICILLIN PRODUCTION	
	Without precursor u./ml.	With precursor u./ml.
Corn-steep solids, 20	650	1300
Cottonseed oil meal, 30	800	1200
Coconut oil meal, 60	450	1150
Rapeseed oil meal, 60	730	705
Groundnut oil meal, 40	1050	1350
Castor bean meal, 20	225	940
Linseed oil meal, 20	700	1035

TABLE 2—ANALYSIS OF OILCAKES

(% on dry basis)

CAKE	MOISTURE	N	OIL	ASH	SILICA
Groundnut	7.99	7.03	7.25	6.52	1.02
	8.98	7.42	6.80	7.20	2.34
	7.04	8.12	9.40	6.63	2.07
	..	8.50	5.50	5.80	..
Castor	..	6.50	7.10	..	..
Mahua, solvent extracted	..	1.81	nil	..	..

TABLE 3—PENICILLIN PRODUCTION USING VARIOUS ADDITIVES

(Medium: Lactose, 3.0; calcium carbonate, 1.0; and sodium sulphate, 0.05 per cent + additive medium indicated in col. 1)

ADDITIVE %	PENICILLIN PRODUCED, u/ml.	
	Bioassay	Chemical assay
Groundnut meal, 2.0 + glycerol, 0.6	1,450-1,580	1,430-1,510
Groundnut meal, 2.0 + lactic acid, 0.6	1,070-1,230	1,050-1,230
Groundnut meal protein, 0.5 + glycerol, 0.6	790-940	830-850
Groundnut meal protein, 0.5 + lactic acid, 0.6	335-480	250-410
Solvent extracted groundnut meal, 2.0 + glycerol, 0.6	1,400-1,480	1,325-1,440
Solvent extracted groundnut meal, 2.0 + glycerol, 0.6 + phenylacetamide, 0.2	1,400-1,496	1,290-1,300
Solvent extracted groundnut meal, 2.0 + lactic acid, 0.6	1,360-1,370	1,340-1,370
Solvent extracted groundnut meal, 2.0 + lactic acid, 0.6 + phenylacetamide, 0.2	1,300-1,320	1,080-1,100
Groundnut meal, 2.0 + glycerol, 0.6 + phenylacetamide, 0.2	1,450-1,500	1,460-1,510
Groundnut meal, 2.0 + lactic acid, 0.6 + phenylacetamide, 0.2	1,300-1,320	1,320-1,350
Corn-steep liquor, 6.0	1,040-1,150	1,050-1,180
Castor cake, 2.0 + glycerol, 0.6	800	..
Solvent extracted castor cake, 2.0 + glycerol, 0.6	800	..
Mahua cake, 2.0 + glycerol, 0.6	285	..
Solvent extracted mahua cake, 2.0 + glycerol, 0.6	285	..

the fermentation was carried on in a shaker at 270 r.p.m. for 120 hr. The samples were then assayed for penicillin, pH and residual lactose.

In some flasks, 0.1 g. of phenyl acetic acid (sodium salt) was added every 24 hr. till the fourth day whereas in the others, only phenylacetamide was added every 24 hr. till the fourth day whereas in the others, only phenylacetamide was added in the beginning. The results of the fermentation studies in the shake flasks are given in Table 3. The precursor (0.1 g.) was added every day unless stated otherwise.

The pH of the medium in all cases was adjusted to 5 before sterilization. The pH at the end of the fermentation at 120 hr. ranged between 7 and 7.5 and the residual lactose varied from 0.2 to 1.2 per cent. Wherever the residual lactose was high the penicillin titres were low.

Some of the experiments were done in 10-litre fermentors by the authors<sup>3</sup> at the Instituto Superiore de Sanita, Rome (1954) with hand-pressed groundnut meal. The penicillin titres were up to 2,000 u./ml. at 96 hr.

It will be seen from Table 3 that of the three oilcakes, groundnut meal is the best for the production of penicillin in conjunction with phenylacetamide

TABLE 4—EFFECT OF ADDITIVES IN PENICILLIN PRODUCTION  
(Medium : Lactose, 3.0 ; calcium carbonate, 1.0 ; sodium sulphate, 0.05 ; and groundnut meal, 2.0 per cent + additive indicated in col. 1)

ADDITIVE %	YIELD* u./ml.
Ammonium acetate, 0.1	805
Potassium acetate, 0.1	873
Sodium acetate, 0.1	900

\* By bioassay

added to the medium in the beginning. But a reference to Table 2 shows that the nitrogen content of castor and mahua cakes is low and therefore larger quantities of these should be used for the fermentation before finally discarding them. The results show that groundnut meal could easily replace corn-steep liquor for the production of penicillin. In general, glycerine containing medium is better than that not containing glycerine. The results in the shake flasks need to be confirmed on pilot-plant scale before they could be tried on large scale manufacture of penicillin.

Martin *et al.*<sup>4</sup> have shown that acetate, formate, and to a less extent lactate, are incorporated into penicillin. The effect of the addition of acetic acid and sodium, potassium and ammonium acetates in the medium for penicillin production was, therefore, studied. The results appear in Table 4. The experiments were done under the same conditions as those recorded in Table 3. Further work is in progress.

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#### Discussion

DR. V. SUBRAHMANYAN: The amino acid make up of groundnut protein is well known. It is a poor source of cystine. Castor cake contains ricin which is poisonous. Mahua cake is not fermentable. If cystine is considered necessary, wood hydrolysate, which is a good source of cystine, could be tried as a supplement to the medium.

DR. P. N. NANDI: The role of trace elements present in different oilcakes should be studied.

DR. R. KAUSHAL: There are many unknown factors responsible for good yields in penicillin production. In view of this it is necessary to screen every oilcake including their proteins, carbohydrates and trace elements present in the ash before any one or more could be used as a constituent of the medium for penicillin production. With this object the work has been undertaken.

It would be worth-while to experiment on using hydrolysate as a supplement to the medium for penicillin production and we shall take it up.



# Utilization of Tamarind Seeds in Penicillin Production

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Suitable preparations of tamarind seeds have been found to be substitutes for corn-steep liquor in penicillin production (1) by the surface culture of *Penicillium chrysogenum* (Thom. NRRL 1951) and (2) by submerged culture of another strain of *P. chrysogenum* (Q-176).

Decorticated tamarind seed powder finds application in many industries<sup>1</sup>, particularly in the textile industry as a sizing agent. Unlike starch, tamarind seed powder has a high protein content (nearly 15-18 per cent) the presence of which makes it susceptible to microbial attack. Desai *et al.*<sup>2</sup> reported that compared to maize starch, tamarind seed powder has slightly higher susceptibility to mildew. This suggested an application of this indigenous material as a source of nutrient for micro-organisms in fermentation industries. In addition to its being an efficient supplement for elaboration of fungal amylase by strains of *Aspergilli* (authors' unpublished work) it has been found that suitable preparations of tamarind seeds could be used as a substitute for corn-steep liquor in the production of penicillin by surface and submerged cultures. The present paper describes some initial experiments in this direction.

## EXPERIMENTAL

Corn-steep liquor was obtained through the courtesy of Messrs *Maize Products Ltd.*, Ahmedabad. Tamarind seed preparations were prepared in the laboratory as follows:

*Tamarind seed powder*—A commercial sample of decorticated tamarind seeds was pulverized in a disintegrator to a mesh size of 120.

*Defatted tamarind seed powder*—The powder obtained as above was solvent extracted with petroleum ether in a Soxhlet extractor (yield of oil, 6.1 per cent). The last traces of the solvent were removed from the powder under vacuum.

*Hydrolysate of tamarind seed powder*—Tamarind seed powder either as such or after defatting could not be incorporated in the medium in high concentrations as it forms a thick viscous paste during autoclaving. Hence the hydrolysate was prepared as follows: 10 g. of tamarind seed powder was

treated with 150 ml. of distilled water containing 1 ml. of pure hydrochloric acid. The mixture was autoclaved under a steam pressure of 15 lb./sq. in. for 1 hr. The hydrolysate was cooled to room temperature and was neutralized to pH 4.5.

*Germinated tamarind seed extract*—Tamarind seeds were placed in moist soil for eight to ten days. The sprouted seeds were cleaned from soil particles and the testa detached. The soft kernels thus obtained were cut into small pieces and steeped in distilled water containing 0.24 per cent sodium sulphite for 48 hr. at 60°C. with occasional shaking according to the procedure of Bowden and Peterson<sup>3</sup>. After steeping process the germinated seed extract was filtered off and concentrated under high vacuum in a falling film evaporator to a total solid content of 40 per cent approx. The pH of the extract was finally adjusted to 4.5 with dil. hydrochloric acid.

The mould strains, *Penicillium chrysogenum* (Thom. NRRL 1951) and *P. chrysogenum* (Q-176) were used for surface and submerged productions respectively. The relative production of the antibiotic was studied using the basal media and methods of Moyer and Coghill<sup>4</sup>. The medium consisting of salts solution, glucose and lactose was supplemented with tamarind seed preparations or corn-steep liquor on the basis of 2 per cent solids. The pH of the medium at the beginning of the experiment was adjusted to 4.5. Fifty-ml. quantities of the medium were distributed in 250-ml. conical flasks. After sterilization, 1 per cent calcium carbonate was added to each flask. The inoculum consisted of 1 ml. of a spore suspension of the culture grown on 5 g. of sterile bread (incubated for 4-5 days at 28°C.) as recommended by Moyer and Coghill<sup>4</sup>. The flasks were incubated at 28°C. for 12-14 days. For submerged production, the flasks were shaken on a reciprocating shaker. The penicillin content was determined against *Micrococcus pyogenes* var. *aureus* (P 209) by the cup-assay of Heatley<sup>5</sup> as modified by Schmidt and Moyer<sup>6</sup>. After four days of incubation, samples were taken out at an interval of 24 hr. The dry weight of the mycelium, pH and the penicillin content of the culture filtrate was determined in each case. Table 1 represents the results on the maximum day of penicillin production under surface conditions using the strain *P. chrysogenum* (Thom. NRRL 1951).

TABLE 1—COMPARATIVE EFFECT OF TAMARIND SEED PREPARATIONS AND CORN-STEEP LIQUOR ON PENICILLIN PRODUCTION\*

(Surface conditions: *P. chrysogenum* Thom. NRRL 1951)

SUPPLEMENT	DAY OF MAX. YIELD	pH OF CULTURE FILTRATE†	MYCELIAL WT.† g./50 ml.	PENICILLIN† %
Tamarind seed powder	11	8.2	0.60	19
do. (defatted)	11	8.3	0.59	18
do. (hydrolysate)	10	8.2	0.76	21
Germinated tamarind seed extract	9	8.1	0.74	98
Corn-steep liquor	7	7.2	0.82	100

\* based on 2 per cent total solids

† on the day of maximum yield

## RESULTS

The results (Table 1) indicate that maximum production of penicillin is obtained with corn-steep liquor medium on the seventh day, while in case of the medium containing tamarind seed preparations antibiotic production was delayed by three to four days. It was also observed that pH of the culture filtrate was slightly higher in the medium supplemented with tamarind seed preparations than in the medium supplemented with corn-steep liquor.

When compared on the basis of 2 per cent total solids, tamarind seed powder either as such or on defatting or hydrolysing proved only about one-fifth as effective as corn-steep liquor in respect of penicillin yields. In attempts to increase the yield of the antibiotic further, it was observed that the extract of germinated tamarind seeds was as effective as corn-steep liquor without the addition of any chemical precursors. Similar results were obtained with another strain of *Penicillium chrysogenum* (Q-176) under submerged conditions of production, penicillin yield with germinated tamarind seed extract being 98 per cent compared to corn-steep liquor (both based on 2 per cent total solids).

Work on the utilization of tamarind seed preparations as a carbohydrate source, effect of precursors and its economic feasibility are in progress.

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# Factors Influencing Pellet Formation in Shake Flask Fermentation

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Hindustan Antibiotics (Private) Ltd., Pimpri

Pellet formation, a characteristic property of fungi growing in submerged cultures, is influenced by several factors. In the commercial strains of *Penicillium chrysogenum* derived from Wisconsin strains, the following factors have been found to be influencing pellet formation in contrast to long mycelium type : (i) age of spores, (ii) number of spores in the inoculum and (iii) rotary or reciprocating type of agitation. The relative influence of each one of the factors on the mycelial type and penicillin yield is discussed.

Factors influencing the type of mycelial formation in shake flask cultures of *Penicillium chrysogenum* Thom. have been studied intensively and results relating to the amount of aeration and agitation influencing the type of mycelial formation have been published<sup>1,2</sup>. The long and short mycelium, sclerotic pellet formation are instances to indicate that aeration and agitation in the fermentors influence the development of one type or the other. The conditions governing pellet formation in submerged cultures in shake flasks have not been completely understood and it was apparent that several factors are involved. The age of the spores for instance appeared to influence the type of mycelial formation and penicillin production in some of the experiments. The amount of spore inoculum was found to be one of the factors either in production of mycelial or pellet formation. It was therefore desirable to study the effect of the age of spores, concentration of spore inoculum as well as the method of agitation (rotary or reciprocating shaker) on the type of mycelial formation and incidentally penicillin yield.

## EXPERIMENTAL AND RESULTS

Experiments were carried out in shake flask cultures and the type of mycelial growth was checked up periodically under the microscope. The age of the spores had to be determined accurately. It was found that on agar slants the mycelium continued to produce new crop of spores for long periods, so that the spores taken from an agar slant were of different ages. In contrast, when sporulated on barley grains, the vegetative hyphae after 8 to 10 days collapsed due to the drying of the grains and the mass of spores was left as

a powdery coating. It was, therefore, possible to obtain spores of same age by this method of sporulation.

Two spore concentrations were used in these studies, one low concentration of 300 spores per ml. and another 30,000 spores per ml. The spore counts were made to obtain the necessary spore concentration in the medium.

The mycelial type and penicillin yield in each flask was checked up periodically and the data finally tabulated at the end of 144 hr. fermentation. The type of mycelial development is given in Table 1.

The results obtained may be summarized as follows.

*Age of spores*—Young spores (10 days old) produce in submerged culture pellets in both high and low spore concentrations when placed on reciprocating shaker, while on rotary shaker pellets are formed when low concentration of spore inoculum is used and chiefly mycelium with few pellets in high spore concentrations.

Medium aged spores (25 days old) produce pellets when low spore concentration is used either on rotary or reciprocating shaker, and high spore concentrations produce mycelium only in rotary shaker and mycelium with pellets in reciprocating shaker.

Old spores (40 days old) produce few large pellets in both the types of shakers when high spore concentration is used and very little growth at low concentrations. This is due to the fact that the effective spore inoculum is reduced either due to failure or delayed germination of the spores.

*Concentration of spores*—Low concentration of spores, irrespective of age and type of shaker, tend to produce pellets. But when the effective inoculum is high, pellets are formed only in young spores agitated in reciprocating shaker, while in 25 days old spores, both mycelium and pellets are formed. On rotary shaker under the above conditions, mycelium and pellets are formed in 10 days old spores and mycelium only in 25 days old spores.

*Shaker*—In both 10 and 25 days old spores at high concentration, the rotary shaker tends to form mycelial forms more than the reciprocating shaker. The mechanical action of the rotary shaker is therefore evident.

TABLE 1—INFLUENCE OF AGE OF SPORES, AMOUNT OF SPORE INOCULUM AND TYPE OF SHAKER ON MYCELIAL TYPE\*

AGE OF SPORES days	SPORES/ML.	ROTARY SHAKER	RECIPROCATING SHAKER
10	300	Pellets	Pellets
	30,000	Mycelium + pellets	Pellets
25	300	Pellets	Pellets
	30,000	Mycelium only	Mycelium + pellets
40	300	Scanty growth	Scanty growth
	30,000	Few big pellets	Few big pellets

\* Strain, *P. chrysogenum* Wis. 47-1564; age of spores, 1, 25 and 40 days, grown on barley grain medium incubated at 24°C.; medium, Lumb's medium with 0.1 per cent antifoam (grapeseed oil); spore concentration, 300 and 30,000 spores per ml.; agitator, rotary shaker at 220 r.p.m. and reciprocating shaker with 90 movements/min.; replicates, 3 replicates of 500 ml. flasks with 125 ml. of media

TABLE 2—INFLUENCE OF AGE OF SPORES AND SHAKER ON PENICILLIN YIELD\*

AGE OF SPORES days	PENICILLIN YIELD, u/ml.	
	Reciprocating shaker	Rotary shaker
10	375	408
25	200	250
40	150	175

\* Strain, Wis. 47-1564 ; medium, Lumb's medium ; spores, 3000 spores per ml. ; replicates, 4

The results of study of the influence of age of spores and shaker on penicillin yield are given in Table 2.

Experiments were carried out in Lumb's media since in this semi-synthetic medium the results are fairly reproducible. Wis. 47-1564 on enriched production media yields up to 1,000 u./ml. of penicillin. The data in Table 2 indicates that maximum penicillin is produced by young spores (10 days old) on rotary shaker.

### DISCUSSION

From the above data the following facts become manifest. In shake flask cultures, the youngest spores have tendency to form pellets and high spore concentration and rotary shaker tend to increase mycelial formation. The rotary shaker influences filamentous mycelial formation more readily than reciprocating shaker. In a previous paper it was shown that in reciprocating shaker there is tendency for loss of spore concentration by the throwing up of mycelia and pellets on the sides of the flask above the level of liquid media. This, however, does not take place at the spore concentration (30,000 spores) used in the present study. The pellet formation observed in the reciprocating shaker at high spore concentration (10 days old) is due to the type of agitation rather than lessening of spore concentration in the liquid media. The age of spores has also been shown to be an important factor in influencing the mycelial type and penicillin yield.

### ACKNOWLEDGMENT

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# Factors Influencing Pellet Formation in Large Fermentors During Penicillin Production

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The type of mycelium developed during different periods of fermentation cycle is under study to correlate mycelial type with penicillin yields. While pellet formation is usually associated with high yields presumably due to larger number of actively growing mycelium, it is responsible for losses during filtration of the broth. The type of growth in fermentor is chiefly determined by the type of growth in seed vessels.

While age and number of spores and the richness of media partly influence pellet formation in seed vessels, it has been found that pretreatment of spores before inoculating seed vessels considerably controls pellet formation. High concentration of spores when germinated on a shaker for 24 hr. and inoculated into seed vessels formed a purely long mycelial type of growth and continued to have similar growth form in the fermentor also. Best filtrations were obtained in all these cases though the yields were somewhat less than in fermentors with pellet formation only.

The type of mycelial development during the fermentation of *Penicillium chrysogenum* is being studied to correlate mycelial type with penicillin yields. Microscopic details like autolysis of hyphal cells, formation of short mycelium swollen or hypertrophied cells and formation of chlamydospore-like bodies often give a better picture of the progress of fermentation than chemical analysis. The type of growth in the fermentors, whether mycelial or pellety is important both for penicillin yield and filtration. When pellety, the broth has granular consistency and during filtration on a rotary drum filter, the mycelium does not form a mat resulting in loss of considerable quantities of broth along with the rejected mycelium. On the contrary, when the mycelium is not pellety, but matty, high vacuum is preserved on the drum resulting in a highly compressed mat with little loss of the penicillin-rich broth. Table 1 presents the relative losses in the two mycelial types during filtration.

While pellety mycelium is disadvantageous from the filtration aspect, high penicillin yield in the fermentor is almost invariably associated with pellety type of growth. This is presumably due to the fact that the total amount of actively growing surface presented in a pellet is several times more than in a long branched mycelium where such activity is restricted to the tips only. However, it is desirable in a plant employing a rotary filter, to have a combination of pellets and long mycelium in the broth to meet the requirements of high yields and efficient filtration.

TABLE 1—MYCELIAL TYPE VS. PENICILLIN YIELD  
(65 hr.)

BATCH NO.	MYCELIAL TYPE	PENICILLIN u./ml.	FILTRATION LOSS %
482	Matty	1480	9.0
487	do.	1430	3.0
511	do.	1440	2.0
513	Pellety	1860	20.2
290	do.	2050	22.0
333	do.	1900	22.0

Detailed studies were made on the factors influencing mycelial or pellet formation during fermentation. It soon became evident that the form of mycelial growth in the 5,000-gal. fermentor is determined by the type of growth of the mycelium in the seed vessel. Once pellet formation is initiated in the seed vessel, the same type of growth is continued in the fermentor to a large extent. Matty or pellety type of growth in the seed vessel is perpetuated in the fermentor.

It is true that the pellety or non-pellety type of growth is typical of the strain also. Thus one of the foreign strains named RS-I never formed pellets, while most of the derivatives of Wis. 51-20 series produced abundant pellets. High yielding derivatives produced by mutation, having restricted heaped growth, tend largely to form pellets. However, the formation exclusively of pellets can be averted by cultural operations. Some of these are presented and discussed.

Growth of HA-2 spores (a derivative of Wis. 51-20) in the seed vessel is as follows: The stout germ tube emerges from the spore after 14 to 16 hr. and is cut off by a septum. The resulting lower cell mostly never develops further, and the stout upper cell by a series of sub-dichotomous branchings forms a radial type of growth gradually forming a central core of compact (plectenchymatous) cells resulting in the pellet initial. From these pellet initials secondary pellets are formed by the incurling peripheral hyphae separating and forming a tangled mass which gradually develops into secondary pellets as previously described by the authors for sclerotic pellet formation<sup>1</sup>.

Stages in the development of RS-1 that does not form pellets showed that the germ tube emerges from the spore in much the same manner as in HA-2 and is cut off by a septum. However, unlike it, both the cells develop further by long thin branches with septa occurring at longer intervals. No radial type of growth and dichotomous branching is observed, instead long divergent secondary branches are formed without the production of pellet initials and a compact central mass of cells. When HA-2 spores were germinated in a two litre flask with 300 cc. of medium, on a rotary shaker at 270 r.p.m., the growth after germination was not confined to the upper cell only. Both the

cells develop long branches reminiscent of the RS-1 in the seed vessel. The secondary branches arise in a divergent manner. After 24 hr. the mycelium is a thick mass without any pellet initials. Based on these observations 10 billion spores (inoculum concentration in a seed vessel) were distributed in three 2-litre flasks germinated into a matty type of mycelium and pooled together at the end of 24 hr. and inoculated into a seed vessel. After 48 hr. growth in the seed vessel, it was transferred into a large 5,000-gal. fermentor. Pellets were not formed in the seed vessel and in the fermentor but the yield in the tank was about 16 per cent less than a corresponding tank with pellety growth. This has been noticed a number of times. It is reasonable to conclude that the type of growth in the seed vessel determines the type of growth in the fermentor. The initial germination type, whether radial and restricted branching of the upper cell after germination or the development of both the cells into a divergent type of growth determines the form of growth during the entire fermentation.

As has been pointed out in the shake flask experiments (in the previous paper) the age and concentration of the spores play a modifying role in determining the amount and nature of pellets and the proportion of pellets and mycelium in the broth. Media of thick consistency like the production medium used for germinating spores in the seed vessel tend to show less pellet formation, possibly due to poorer aeration condition (indicated by a slower rate of growth) than in the thinner, usual seed media of corn-steep solids and sucrose only.

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# Production of an Antibiotic Substance from a Strain of *Streptomyces* Sp., Ac<sub>3</sub> (203)

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An antibiotic-producing organism belonging to *Streptomyces* sp., designated Ac<sub>3</sub> (203) has been isolated and found to exhibit a marked anti-bacterial action against Gram-positive, Gram-negative and Acid-fast bacteria. The morphological and cultural studies of the isolate, some of the physiological activities of the strain, method of isolation of the active principle and some physico-chemical and anti-microbial properties of the purified product have been described.

In the course of our investigation on the occurrence and isolation of antibiotic-producing organisms from the soils of West Bengal, an isolate belonging to *Streptomyces* sp., designated Ac<sub>3</sub> (203), was found to exhibit a marked anti-bacterial action against Gram-positive, Gram-negative and Acid-fast bacteria. The present investigation deals with a description of morphological and cultural studies of the isolate, some of the physiological activities of the strain, method of isolation of the active principle and some physico-chemical and anti-microbial properties of the purified product.

## EXPERIMENTAL

### Media for production of antibiotic substance

The following media were selected for studies on the production of antibiotic elaborated by Ac<sub>3</sub> (203): (i) Yeast-extract dextrose broth, (ii) Straw-infusion broth<sup>1</sup>, (iii) Neomycin producing medium of Waksman and Harris<sup>2</sup>, (iv) Streptomycin-producing medium of LePage and Campbell<sup>3</sup> and (v) Basal medium of Gottlieb and Pridham<sup>4</sup>.

Yeast-extract dextrose broth had the following composition: Bacto yeast-extract, 2 g. ; dextrose, 10 g. ; water, 1,000 ml., pH, 6 - 6.4.

### Methods of assay

Agar-cup and agar-streak methods of assay were adopted all through the experiment.

### Bio-autographic technique

The technique used as an analytical method for testing the homogeneity of

a very small amount of material consists in developing a paper-chromatogram in suitable solvents. The papers are then dried, placed on assay trays containing media seeded with test organisms, incubated overnight and from the halo-zones,  $R_f$  values are calculated using individual substances as well as their mixtures for resolution. The substance appears to be homogeneous, when single spot is obtained.  $R_f$  value is calculated from the ratio of the distance travelled by the solvent front and the movement of the spot of the substance on paper.

Whatman No. 1 filter paper was used as support for the chromatogram in these investigations. The solvents used for development were wet *n*-butanol, piperidine (2 per cent), *p*-toluene sulphonic acid (2 per cent) and methanol-water (9:1).

### Methods for study of effect of trace elements on antibiotic production

Only Jena and Hysil glasswares were used. To avoid carryover of trace elements in the organic nutrients, the basal medium of Gottlieb and Pridham<sup>4</sup> was employed. The use of a chemically defined medium led to a generally lowered antibiotic titre. It, however, provided a scope for correct assessment of the roles played by individual trace elements with respect to growth and antibiotic production of the strain. It is well known that the presence of even infinitesimal amounts of trace elements would have marked effect on the desired result. Hence only analytical grade of reagents were used as constituents of the medium. The medium was freed from traces of zinc, iron and copper by shaking 750 ml. chloroform (E. Merck) in a separating funnel at pH 7.4 and then again at pH 5.2. After each extraction, the solution was washed thrice with 5 ml. chloroform. The medium was finally washed three times with 5 ml. chloroform and once with 10 ml. chloroform to free it from traces of 8-hydroxyquinoline. After purification, each element was added in requisite amount. The trace element under investigation was added in graded doses, while other elements were added in concentrations as given in the composition of the basal medium.

The final medium was distributed in 100 ml. flasks, 20 ml. in each. The flasks were then sterilized at 15 lb./sq.in. for 15 min. and incubated for 8 days at 28°C., after inoculation with spore-suspensions of the strain *Ac*<sub>3</sub> (203) in water. For the preparation of the inoculum, the culture was grown in 50 ml. of basal medium in 500 ml. flasks for 8 days at 28°C. and the culture-filtrate was decanted. The mycelial felt was washed thrice with sterile water and then shaken with 50 ml. of water. 2 ml. of the spore suspension was added to each 100 ml. flask. The whole operation was carried out under aseptic condition.

After the fermentation was over, the culture-filtrate was assayed against *Escherichia coli* by agar-cup method and the zone of inhibition was measured up to the nearest mm.

The weight of the mycelium was determined by filtering the pellicles through weighed Whatman No. 1 filter paper in Buchner funnel. The papers were dried overnight at 80°C. and then weighed.

TABLE 1—ANTIBIOTIC ACTIVITY OF *Ac*<sub>3</sub>(203) AGAINST BACTERIA AND FUNGI

TEST ORGANISMS	ZONE OF INHIBITION ON AGAR PLATE, mm.	
	Yeast extract dextrose agar	Nutrient agar
<i>Escherichia coli</i>	26	28
<i>Escherichia coli</i> R*	20	18
<i>Bacillus cereus</i> var. <i>mycoides</i>	Not tested	28
<i>Vibrio cholerae</i>	25	20
<i>Eberthella typhosa</i>	28	29
<i>Bacillus pyocyaneus</i>	?	—
<i>Proteus vulgaris</i>	?	28
<i>Staphylococcus aureus</i>	15	24
<i>Bacillus subtilis</i>	Not tested	24
<i>Escherichia coli</i> W†	Not tested	21
<i>Aspergillus niger</i>	—	—
<i>Pythium</i> sp.	—	—

\* *E. coli* resistant to streptomycin ; † *E. coli* resistant to streptothricin ; ? doubtful results ; — no activity

TABLE 2—COMPARATIVE RESULTS OF ASSAY OF ANTIBIOTICS PRODUCED BY *Ac*<sub>3</sub>(203) AND *STREPTOMYCES* *GRISEUS*

TEST ORGANISMS	ZONE OF INHIBITION ON AGAR PLATE, mm.	
	<i>Ac</i> <sub>3</sub> (203)	<i>S. griseus</i> (ATCC 10137)
<i>B. subtilis</i>	24	21
<i>S. aureus</i>	25	17
<i>B. mycoides</i>	28	21
<i>E. coli</i> R*	18-19	..

\* *E. coli* resistant to streptomycin

## RESULTS

The strain *Ac*<sub>3</sub> (203) belonging to *Streptomyces* sp. was selected for further study for the production of antibiotic substance. It showed considerable antagonistic activity against Gram-positive, Gram-negative and Acid-fast bacteria when tested by agar-streak method. The media selected for assay were yeast-extract dextrose agar and nutrient agar. The zone of inhibition produced by an 8-day old culture was measured up to the nearest mm. Table 1 shows the results of screening against 12 test organisms by agar-streak method. Zones of inhibition showed practically no change even on prolonged incubation.



The active principle elaborated by the strain  $Ac_3$  (203) was found to be different from streptomycin, an antibiotic produced by *S. griseus* (ATCC 10137), when tested by agar-streak method of assay under identical conditions (Table 2).

The strain  $AC_3$  (203) was found to elaborate antibiotic substance when grown in liquid media and tested by agar-cup method against *E. coli*.

#### MORPHOLOGICAL AND CULTURAL CHARACTERISTICS OF $AC_3$ (203)

A detailed morphological and cultural study of the strain was taken up in order to identify it with the aid of Bergey's Manual of Determinative Bacteriology. A characteristic description is given below from the collected data.

**Aerial mycelium:** Slender branching unicellular mycelium with spiral formation in sporulating hyphae; filament  $0.7 \times 1.3 \mu$  in diam. Conidia globose to ellipsoidal in form  $1.3 \times 2$  to  $1.3 \times 2.6 \mu$ .

**Nutrient agar:** Good growth; aerial mycelium white; no soluble pigment

**Synthetic agar:** Growth moderate; white, thin mycelium; no soluble pigment

**Potato-beef-extract agar:** Profuse cotton-like growth, white mycelium; faint yellowish pigment

**Potato slant:** Growth profuse; aerial mycelium at first white, then turned grey; pigment light buff

**Czapek-Dox:** Growth profuse; pinkish white mycelium; reverse reddish-brown; soluble reddish-brown pigment

**Organic media:** Growth profuse; greyish mycelium, faint yellow soluble pigment

**Ca malate:** Growth moderate; greyish-white mycelium; no soluble pigment

**Blood agar:** Growth good; complete haemolysis

**Starch:** Grey mycelium with a pinkish tinge; growth profuse; pinkish pigment; not hydrolysed up to third week

**Ordinary milk:** Completely peptonized

**Litmus milk:** Slightly alkaline

**Glucose agar:** White sparse growth with ash-coloured patches; no pigment

**Peptone agar:** White sparse growth; no soluble pigment

**Peptone broth:** Good growth; no pigment; white crust at the top

**Glucose broth:** Thin white deposit at the bottom; no pigment

**Glucose-asparagine solution:** White powdery deposit; no soluble pigment

**Loeffler's serum:** Growth good, wrinkled; white mycelium; reverse yellowish; liquefaction doubtful

**Nitrate broth:** Nitrate not reduced

**Gelatin:** White mycelium; no soluble pigment; complete liquefaction at the end of third week

**Dorset agar:** Moderate growth; not liquefied; mycelium white but turned grey later

**Hydrogen sulphide:** Not produced

Habitat: Indian soil

Optimum temperature: 28°C.

On comparison of the colour produced due to the growth on potato slant with Ridgway's colour standard (1912) the soluble pigment was found to resemble Plate No. XXIX or XL i.e., coloured light pinkish cinnamon or vinaceous buff respectively.

Differential tests were conducted on the basis of carbohydrate utilization for growth<sup>5</sup>. *d*-Glucose, *d*-mannose, cellobiose, starch, dextrin and glycerol were not tested since all the 23 strains in Pridham and Gottlieb's study gave positive results with these C-sources. Table 3 shows the results of these tests with *Streptomyces fradiae*, *Streptomyces lavendulae* and *Ac*<sub>3</sub> (203).

Thus the strain was found to resemble *S. fradiae*, *Streptomyces californicus* 8-80 group in certain respects, although it showed differences in its morphology, some cultural characteristics and capacity for carbon utilization. Hence the strain should claim its recognition as a species related to 8-80 group in Gottlieb and Pridham's classification.

### Production media

Four media were tried for the production of antibiotic substance by the strain, viz., yeast ext. sucrose-broth, straw-infusion broth, neomycin producing medium and streptomycin producing medium.

TABLE 3—GROWTH OF *Ac*<sub>3</sub>(203) ON SYNTHETIC MEDIUM OF PRIDHAM AND GOTTLIEB WITH VARIOUS C-SOURCES

COMPOUNDS	<i>S. fradiae</i>	<i>S. lavendulae</i>	<i>Ac</i> <sub>3</sub> (203)
<i>l</i> -xylose	—	—	+
<i>l</i> -arabinose	—	—	fair
Rhamnose	+	—	+
<i>d</i> -galactose	+	+	+
Sucrose	+	—	(—)
Maltose	+	+	+
Raffinose	+	—	+
<i>d</i> -sorbitol	+	(—)	—
Dulcitol	+	—	—
Mannitol	+	(—)	—
Lactose	+	—	+
Sodium acetate	+	(—)	+
Sodium succinate	+	+	+
Sodium citrate	+	+	+
Fructose	+	?	+

+ growth and positive utilization; — no growth and no utilization; (—) feeble growth and probably no utilization; ? variable reaction

TABLE 4—ANTIBIOTIC TITRE OF CULTURE FILTRATES

	ZONE OF INHIBITION, mm.	
	Undiluted	1:10 dilution
Yeast ext.-sucrose broth	14	—
Straw-infusion broth	20-21	14
Neomycin-producing medium	—	—
Streptomycin-producing medium	11-12	—

— No inhibition

TABLE 5—EFFECT OF GLUCOSE AND CALCIUM CARBONATE ON ANTIBIOTIC TITRE

No.	GLUCOSE g.	CALCIUM CARBONATE g.	ZONE DIAM. WITH <i>S. aureus</i> , mm.	
			4th day	8th day
1.	1	0	12	22
	1	0.75	11-12	20-21
	1	1.50	..	20
2.	2	0	16	22
	2	0.75	13-14	24-25
	2	0.50	13-14	24-25
3.	4	0	..	22
	4	0.75	20	19
	4	1.50	..	18

The strain was grown in 50 ml. of each medium under stationary conditions in 250 ml. Erlenmeyer flasks. After an incubation for 8 days at 28-30°C., the culture-filtrate was assayed against *Eb. typhosa* by agar-cup method. Results (Table 4) show that straw infusion broth was superior to other preparations from the stand-point of antibiotic production. Straw-infusion-dextrose broth was therefore selected as production medium. The addition of 0.2 per cent agar facilitated the growth and raised the antibiotic titre but the method was abandoned later on due to subsequent difficulties in the method of extraction.

The effect of addition of glucose and calcium carbonate was also studied. Fifty ml. quantities of the straw-infusion medium were taken in 250 ml. Erlenmeyer flasks, to which glucose and CaCO<sub>3</sub> were added in graded doses. The culture flasks were incubated at 28°C. for 8 days and at the end of the fourth and eighth day the filtrate was assayed against *S. aureus*. by agar-cup method and zones of inhibition were measured. The results are given in Table 5. It appears from the table that the addition of 2 per cent glucose



TABLE 6—IN VITRO ANTI-MICROBIAL SPECTRUM OF PURIFIED PRODUCT

TEST ORGANISMS	DILUTION UNITS/G. OF PURIFIED MATERIAL
<i>Aerobacter aerogenes</i>	15,000
<i>B. mycoides</i>	6,000
<i>B. subtilis</i>	6,250
<i>Corynebacterium diphtheriae</i>	5,000
<i>E. coli</i>	12,000
<i>Mycobacterium phlei</i>	10,000
<i>P. vulgaris</i>	3,000
<i>S. aureus</i>	6,000
<i>V. cholerae</i>	5,000

and 0.75 per cent calcium carbonate to the straw-infusion medium gives the highest antibiotic titre at the end of the eighth day.

The pH of the medium changed from 7.4 to 6.4 at the end of the eighth day of incubation.

### Recovery and purification of the active principle

The method of purification resembled that of streptomycin and streptothricin<sup>6,7</sup>. The cultures were grown in 100 ml. of straw infusion broth under stationary condition in Roux bottles for 8 days at 28°C. and filtered. The mycelial felt was washed with acid-water and adjusted to pH 2.5. The culture filtrate, to which the mycelial wash-liquor was added, was absorbed on 2 per cent Norit charcoal (SB × 30). The charcoal was air-dried and then eluted by means of 80 per cent acetone acidified to a pH 2.5 with sulphuric acid (2N). The acetone was removed by evaporation under vacuo and precipitation was effected by the addition of 5 vols. of acetone to the residual solution. The precipitate, thus obtained, was kept overnight at 4°C. and centrifuged. The centrifuged mass was dissolved in the minimum amount of water and reprecipitation was effected by absolute alcohol. The operation was repeated thrice, the final precipitate was centrifuged and dried under vacuo which gave a dirty powdery mass.

### Properties

The *in vitro* anti-microbial spectrum of the purified product is shown in Table 6.

A comparison of the antibacterial action of the active principle against *E. coli* with that against different bacteria will be evident from Table 7. The Table also shows in a comparative way the same ratio, when neomycin, and components thereof, were used as antibiotic substances.

The substance was readily soluble in water but insoluble in most organic solvents which included among others, ether, *n*-butanol, benzene, ethylene-dichloride and ethanol.

The stability of the antibiotic substance in presence of cysteine was then determined. The purified product was kept in presence of 1.0 mg. cysteine at 25°C. and the antibiotic action was tested at different time intervals against *E. coli* by agar-cup method. Table 8 shows that the substance was not inactivated in presence of cysteine, even when kept for 24 hr. at 25°C.

The substance was found to be less stable in the alkaline range.

The effect of temperature on the activity of the purified product has been shown in Table 9.

The results indicate that the substance was thermostable to a considerable extent. The substance did not lose its property when kept in dry condition for more than 6 months at room temperature. In solution, however, the activity was considerably diminished.

TABLE 7—RATIO OF DILUTION ASSAY AGAINST VARIOUS BACTERIA TO DILUTION ASSAY AGAINST *E. COLI*

TEST ORGANISMS	Ac <sub>3</sub> (203)	NEOMYCIN (crude)	VARIOUS COMPONENTS OF NEOMYCIN COMPLEX*		
			1	1	1
<i>E. coli</i>	1.00	1.0	1	1	1
<i>B. subtilis</i>	0.45	11.0	11	10	16.7
<i>S. aureus</i>	0.50	3.6	2.4	3.3	1
<i>B. mycoides</i>	0.50	..	..	..	..

\* Waksman et al.

TABLE 8—EFFECT OF CYSTEINE ON ANTIBIOTIC SUBSTANCE

TIME hr.	ZONE OF INHIBITION ( <i>E. coli</i> ) mm.
0	24-25
3	24
6	23-24
12	22-23
24	23

TABLE 9—EFFECT OF TEMPERATURE ON THE ACTIVITY OF Ac<sub>3</sub>(203)

TEST MATERIAL	ZONE DIAM. ( <i>E. coli</i> ) mm.
Original solution (control at room temp. 28°C.)	21-22
Solution kept for 15 min. at 100°C.	20
Solution kept for 30 min. at 100°C.	20

The substance gave negative tests with ferric chloride, 2-4-dinitrophenylhydrazine and Fehling's solution.

The purified product was analysed as sulphate after drying at 60°C. under vacuo.

The ultraviolet spectrophotometric analysis showed absorption in the end spectrum.

The infra-red studies in both cyclohexanol and nujol indicated -NC bonds and the pattern showed polypeptide nature of the compound. Since the substance was found to be a polypeptide in nature it was hydrolysed with 6N HCl in a sealed tube for 24 hr. at 120°C. and the hydrolysate was analysed by means of paper chromatography, both one-dimensional and two-dimensional, using phenol-water and benzyl alcohol and acetone acid-water (25:5:6.5) as the developing solvents. Ninhydrin was used as spraying reagent, seven to eight spots (one doubtful) appeared on the chromatogram with the following  $R_f$  values: 0.11, 0.12, 0.14, 0.19, 0.24, 0.32, 0.52 and probably 0.04.

The properties of the substance indicated its resemblance with antibiotics belonging to the less characterized group 'Neomycin Complex'. Attempt was therefore made to identify the substance with the help of the recently developed bio-autographic technique<sup>8</sup>. From the halo-zones,  $R_f$  values were calculated, using individual substances as well as their mixtures (Table 10). The substance appeared to be homogeneous as a single spot was obtained when tested by means of bio-autographic technique. Preliminary toxicity tests with white Swiss mice (body weight 20 g.) indicated that intramuscular injections of 120-150 dilution units were tolerated.

The antibiotic Ac<sub>3</sub> (203) was studied by a 24-tube counter-current distribution apparatus set up in this laboratory. The solvents selected were KCl-boric acid buffer (pH 7.8) and amyl alcohol-5 per cent stearic acid mixture. After shaking for 5 min. for each operation and allowing 10 min. for equilibrium to be attained, the substance was distributed in the 24-tubes and analysed. To each tube were added 1 or 2 drops of HCl and 5 ml. chloroform and the tubes were shaken for 10 min. or more. Finally 2 ml. of solution from the aqueous layer were taken out, evaporated to dryness under vacuum and weighed in tared weighing bottles specially designed for the purpose. The weight obtained was plotted against the corresponding tube number.

A critical analysis of the curve shows that the substance exhibited three peaks at least for the solvent system selected. In the case of 24 tube distribution the

TABLE 10— $R_f$  VALUES OF VARIOUS ANTIBIOTICS AND THE TEST MATERIAL

ANTIBIOTIC	SOLVENT	$R_f$ VALUE
Neomycin	1. Wet <i>n</i> -butanol + piperidine, 2% + <i>p</i> -toluene sulphonic acid, 2%	0
Chloromycetin		0.91
Test material		0
Neomycin	2. Methanol: water (9:1)	0
Test material		0.14



sharp fall from 0 to 2 was possibly due to the presence of impurity (residual matter as shown by micro analysis) in the sample. The antibiotic from the tube 18 was extracted, purified and a sample has been sent to Dis Weiler and Strauss (Oxford) for micro-analysis in order to verify the chemical composition of the purified product.

It is now well-known that a substance may not be homogeneous even if one peak appears in the distribution pattern. Homogeneity is proved only when positive results are obtained by other tests as also in other solvent systems.

It is expected that further study on counter-current distribution using different solvent systems and larger number of tubes may reveal the presence of allied substances in the purified sample.

It is also contemplated that the acid hydrolysate of the antibiotic which contains a number of amino acids as shown by paper chromatography be distributed between HCl-Phenol phases after degradation with 6N HCl for different time periods so that the sequence of amino acids may be studied.

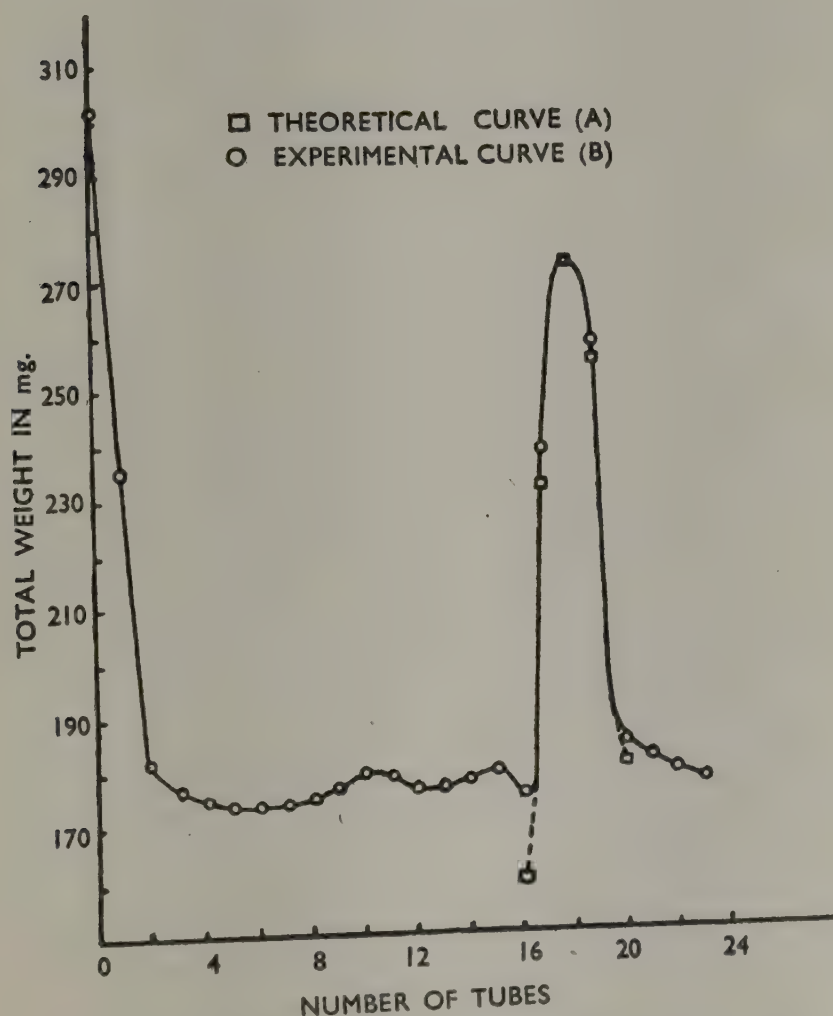


FIG 1—DISTRIBUTION PATTERN OF THE ANTIBIOTIC  $Ac_3$  (203)

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# Production of Mutants in *Penicillium notatum* Induced by Ultra-violet Irradiation

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Conidial suspensions of *Penicillium notatum* in sterile solution (1 : 10,000) of a wetting agent, 'Calsolene', have been subjected to ultra-violet irradiation. Out of 250 isolates, 164 mutants proved to be different from the parent strain in penicillin output and in morphological characteristics in certain cases.

Of the 164 mutants, definite change in morphology was observed in 57 isolates. Eighteen produced the same amount of penicillin as the parent, 32 produced lesser and 7 no penicillin at all. However, none of the morphologically altered cultures showed any increased amount of penicillin production in comparison with the parent strain.

It is now generally accepted that ultraviolet radiation could possibly be used as a means of developing substrains of antibiotic producing fungi. Extensive programme of irradiation was undertaken in the U.S.A.<sup>1-6</sup> to develop better strains of *P. notatum* for the penicillin industry. The technique has been utilized successfully for better production of organic acids from moulds by fermentation while irradiation produced large number of mutants of *Neurospora* sp. having altered biochemical properties. The technique of producing mould mutations artificially was thus well established and it seemed reasonable to believe that mutations might be produced in an organism in which the biochemical sequence is likely to be altered distinctly, resulting in a possible change in the accumulation of the metabolic products. The present investigation is an attempt to develop high-yielding strains of *P. notatum* from a locally isolated culture. It gave 50 units of penicillin/ml. when grown in Higuchi's<sup>7</sup> medium by the surface culture method.

## EXPERIMENTAL

A Phillips germicidal lamp of 30 W. capacity was used for irradiation. This lamp was designed in such a way as to emit 90 per cent of the rays having wave-length at 2537Å which is the bactericidal range. Conidial suspensions were prepared in sterile solution of a wetting agent 'calsolene' (1 : 10,000) from (7 days old) agar plates and the spores were thoroughly dispersed by shaking. The depth of the liquid was maintained at 2-3 mm. in a flat bottomed uncovered sterile Petri dish and the suspension was stirred all through the irradiation by a bent glass rod. Samples were removed at intervals of



TABLE 1—GROUPS OF MUTANTS OBTAINED

RUN	COLONIES ISOLATED	CULTURES SELECTED FOR DETAILED STUDY
1	45	25
2	60	36
3	40	20
4	56	42
5	49	41

2 min. up to 10 min. Dilutions were made at desired steps and plated out in Czapek-Dox agar. The plates were incubated at 25°C. for 4-5 days, random isolations were made from the developing colonies and were tested in stationary cultures to determine their capacity to produce penicillin. Fifty ml. quantities of Higuchi's medium were distributed in 250 ml. conical flasks and incubated at 25°C. Assays were made against *Staphylococcus aureus* as test organism.

### RESULTS AND DISCUSSION

Although 250 isolates were obtained out of those appearing on the culture plates in a set of experiment, only 164 were subjected to detailed study. Table 1 shows the groups of mutants obtained from different runs of the experiments.

The parent strain of *P. notatum* grew fairly well on Czapek-Dox agar and had a close-textured basal felt bearing abundant conidia. The colour of the conidia was bluish green with yellow pigment produced on the reverse.

Certain changes were observed in the isolates arising from the irradiated conidia.

- (i) Colonies unlike the parent in growth and texture, having leathery or much wrinkled appearance with restricted growth and blue green to greenish white, dirty white to pale blue conidial areas.
- (ii) Colonies like parent in growth and texture but with conidial areas of other than blue green shades such as grey, light green, greenish white, yellowish green and pale blue.

In both the above types there were strains which differed from the parent in the change of intensity of the yellow pigment having various gradations.

### Penicillin production

Fig. 1 shows the distribution of mutants as regards penicillin production among 164 strains. They have been divided into the following sub-groups.

- (i) 7 morphologically altered strains where penicillin production was absent.
- (ii) 32 strains which were morphologically altered and produced less penicillin.
- (iii) 18 morphologically altered strains which were equally efficient as the parent in penicillin production.

- (iv) 6 strains which were morphologically unchanged but had lost the property of synthesising penicillin.
- (v) 86 strains which were morphologically unchanged but produced less penicillin.
- (vi) 15 strains which were unaltered morphologically but produced more penicillin.

It is evident that as a result of mutation there have been distinct changes in the genetic constitution of some of the isolates resulting in the alteration of their biochemical activity. Studies on the deficiency mutant will be taken up later.

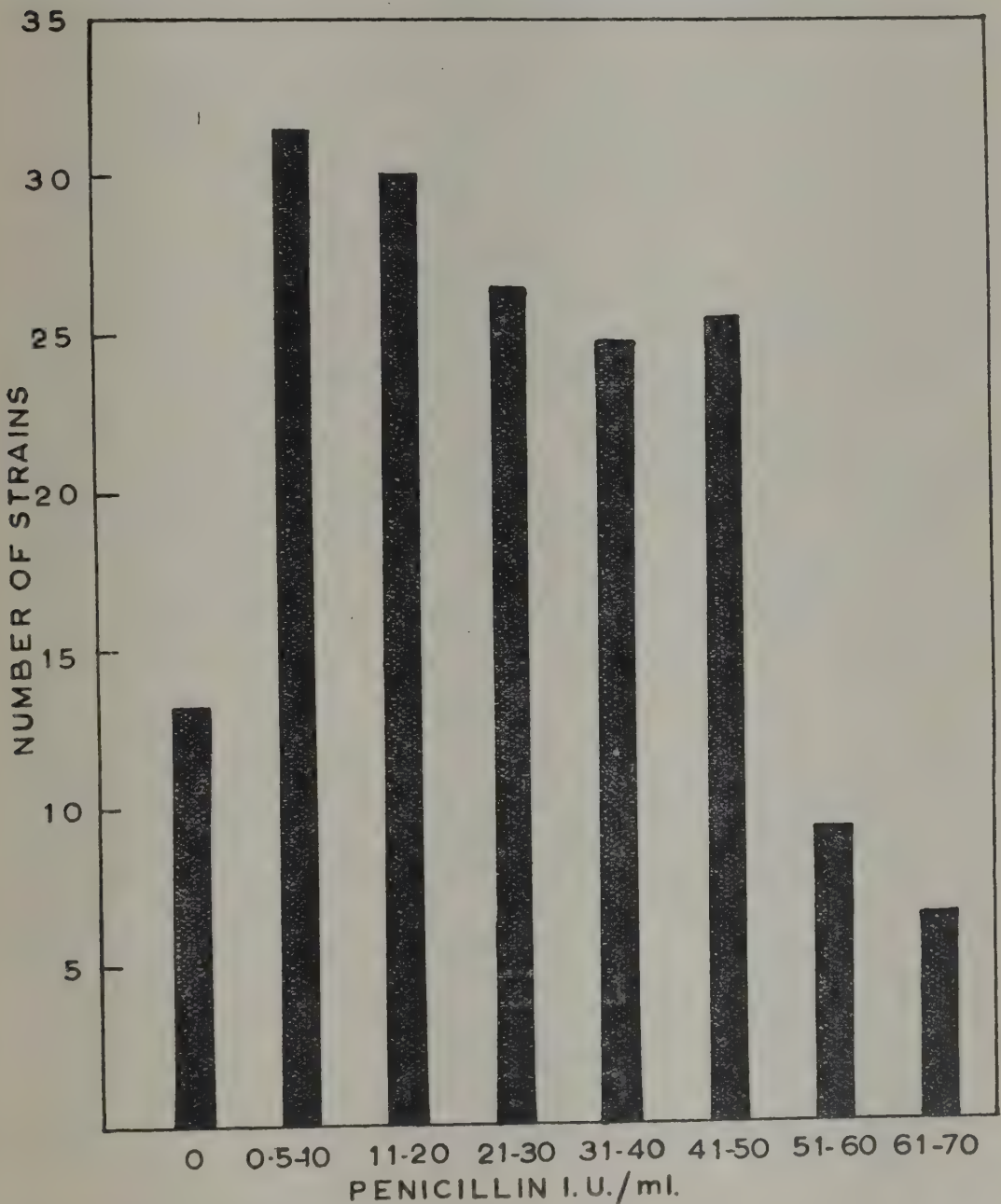


FIG.1—DISTRIBUTION OF MUTANTS BASED ON PENICILLIN PRODUCTION

The success of developing mutants of *P. notatum-chrysogenum* was tested in the case of organisms (*Aspergillus niger*) producing citric acid on a commercial scale. Similar results were also obtained in the case of itaconic acid<sup>3</sup> produced by *Aspergillus terreus*. The work on *Neurospora* sp.<sup>8,9</sup> brought a new era in the field of biochemical synthesis and enormous array of literature has been accumulated on the subject during recent years. However, the course of biosynthesis of any compound can now be studied by considering the gene-enzyme blocks which may occur at varying steps in the course of biosynthesis of an organism. The loss of certain property in mutants can be explained on the basis of a loss of a specific enzyme which controls the reaction concerned. In certain cases a new character appears in a mutant and sometimes an intermediate or an unknown substance is synthesized during the course of metabolic activity.

Lastly, the case of increased production of penicillin or other substances by mutant strains may be considered from another point of view where no new product is formed but there is only increased intensity of production of a substance normally produced by the parent.

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#### Discussion

MAJ.-GEN. S. S. SOKHEY: The author should have tried with a strain producing at least 1,500 units per ml. instead of one producing only 50 units.

DR. P. N. NANDI: The work has been carried out with the aim that experience gained in this work may be profitably used with respect to better penicillin yielding strains.

COL. S. S. BHATNAGAR: Has the author any experience with mutation of other fungi?

DR. P. N. NANDI: Work is being carried out with streptomycetes, but the results obtained so far are not satisfactory.

DR. S. K. BOSE: Is not the percentage of mutants obtained too high?

DR. P. N. NANDI: I have reported just what I am getting with my apparatus.



# Improvements in Technique for Selecting *Penicillium chrysogenum* Strains for Increased Penicillin Yield

M. J. THIRUMALACHAR

Hindustan Antibiotics (Private) Ltd., Pimpri

A method is described for screening mutants of *P. chrysogenum* strains for increased penicillin yield in Petri dishes before selecting strains for shake flask tests. Using the test organism *Xanthomonas malvacearum* and *X. alysycarpi-vaginalis* which show sensitivity to penicillin at 500 units and above, most of the low-yielding strains may be eliminated before taking them to the shake flask testing stage. The testing in the shake flasks can be done more efficiently within a short period.

The development of high yielding strains for penicillin production has been the object of detailed studies by numerous investigators and several valuable contributions have been made. Starting from NRRL 1951-B25 strain which yielded about 200 units of penicillin per ml. the present-day strains have been developed yielding 2,000 to 3,000 units depending upon the fermentation cycle. While improvements in fermentation technique have played some part, the main contribution for increasing penicillin yields has been due to the development of high yielding strains.

In developing high yielding strains, the genetic variability of the fungus induced by mutagenic agents has been exploited to the best advantage. X-rays, ultra-violet rays and nitrogen mustard are most commonly used. In many cases, selection of naturally occurring variants have yielded excellent results. In the case of induced mutagenic agents like X-rays, ultra-violet radiation and nitrogen mustard, the colonies surviving after the treatment are selected and tested for penicillin yield.

In testing the strains for penicillin yield, various techniques have been employed. At the time when the yields of strains were less than 200 units of penicillin, Raper *et al.*<sup>1</sup> described the technique of growing the colonies on agar and cutting a disc of agar along the margin of the colony and testing the penicillin content in the agar disc piece. This is done by placing the agar disc on nutrient agar seeded with the test organism *Bacillus subtilis*. The diameter of the zone of inhibition gives a measure of the amount of penicillin in the agar disc and incidentally the potency of the strain producing it. Since *B. subtilis* is quite sensitive to penicillin and even 10 units give large zone

of inhibition, this technique is not useful when the strain produces very high yields. Secondly, the test is not for the total amount of penicillin produced by the strain because, if the discs of agar are not cut at the same distance from the margin of the colony or if the discs are not of uniform thickness, the variations in results are considerable.

An excellent review of the strain development programme started at the University of Wisconsin in 1945 and continued for over a period of 10 years has been given by Backus and Stauffer<sup>2</sup>. The numerous mutants were selected either after treatment with ultra-violet radiation or nitrogen mustard. A large number of naturally occurring variants were also studied. In each case, the mutants were evaluated by shake flask tests in triplicates, and the best yielders retested in replicates of 10 before taking them to the pilot plant stage. When large number of mutants have to be tested, their evaluation by shake flask-tests becomes a time consuming process requiring lot of equipment and space. If a method could be devised for screening out some of the low-yielding strains, considerable progress could be made in a shorter space of time. The basis for the new technique described in the present paper is a result of previous studies by the author *et al.*<sup>3</sup> on the effect of some commercially available antibiotics on bacterial plant pathogens.

In the studies referred to above, the effects *in vitro* of 5 commercially available antibiotics, viz., aureomycin, terramycin, dihydrostreptomycin, chloromycetin and sodium penicillin G were tested on one large group of 32 species of *Xanthomonas* occurring in India. The first four antibiotics, aureomycin, terramycin, dihydrostreptomycin and chloromycetin in concentrations of 20 to 60  $\mu\text{g.}/\text{ml.}$  completely inhibited all the *Xanthomonas* species. Penicillin had no effect in the concentration of 50  $\mu\text{g.}/\text{ml.}$  used in the test. Following this, the 32 *Xanthomonas* species were tested with high concentrations of penicillin from 250 to 1,000 units per ml. Of the 32 species tested, 20 species showed no reaction to penicillin even at concentration of 1,000 units per ml. and other 12 showed varying degree of sensitivity. The reaction of five *Xanthomonas* species is given in Table 1.

The above data indicate that the organisms mentioned are sensitive to penicillin only at high concentrations. Of these, *X. malvacearum* is most sensitive and *X. alysicarpi-vaginalis* is least sensitive.

TABLE 1—PENICILLIN SENSITIVITY OF XANTHOMONAS SPECIES

	INHIBITION ZONE IN MM. AT DIFFERENT PENICILLIN CONCENTRATIONS		
	250 u./ml.	500 u./ml.	1,000 u./ml.
<i>X. malvacearum</i>	10	20	32
<i>X. cassiae</i>	0	15	26
<i>X. bilvae</i>	12	16	24
<i>X. mehusii</i>	0	12	14
<i>X. alysicarpi-vaginalis</i>	0	0	13

TABLE 2—USE OF *X. MALVACEARUM* IN SCREENING HIGH  
PENICILLIN YIELDING STRAINS

PENICILLIN STRAIN	ZONE mm.	YIELD IN SHAKE FLASK u/ml.
Wis. 47-1564	0	800
Wis. 49-133	0	1,000
Wis. 51-20C	12	1,600-1,800
HA-2	14	1,600-2,000
s-36	20	2,000 and above
A-73	30	2,000 on 2 per cent lactose
HA-3	35	3,000

Following these observations, the two species were used as test organisms for evaluating the penicillin yield of strains. Large number of mutants were obtained by subjecting spores to ultra-violet radiation from a Hanovia germicidal lamp. The time of exposure and distance was adjusted to obtain 20 per cent survivals after irradiation. The various types of mutants with different types of colony characters, yellow and white-spored forms, non-sporulating types, etc. were selected and aseptically transferred to 10 cm. Petri plates with 20 ml. of modified Moyer's agar (lactose,  $1\frac{1}{2}$  and sucrose,  $1\frac{1}{2}$  per cent in place of 3 per cent sucrose only). In each Petri dish 4 colonies were plated at four corners equidistant from each other. The plates were incubated at 24°C. for 5 days after which they were flooded with a 48 hr. old suspension of *X. malvacearum*. The plates were incubated again at room temperature for 36 hr. after which the readings for the zones of inhibition were taken. The thick yellow mass of bacterium formed a sharp zone of inhibition. Some of the data obtained are given in Table 2.

The data of only a few strains have been given. Many of the strains have not been tested on a large scale in shake flasks or the proper fermentation conditions have not been investigated. It therefore appeared that the method of testing the mutants first on agar plates using *X. malvacearum* as test organism enables us to eliminate many of the low yielding strains, so that the testing in the shake flask may be done more efficiently within a shorter period.

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#### Discussion

DR. P. N. NANDI: Can *Xanthomonas* be replaced by other known human pathogens, e.g., *Escherichia coli*?



DR. M. J. THIRUMALACHAR: Tests with *E. coli* and other penicillin-resistant organisms were tried but these were not found useful.

DR. M. B. SAHASRABUDHE: Which U.V. lamp was used for producing mutation?

DR. M. J. THIRUMALACHAR: Germicidal lamps giving the wave length of 2575 Å were used.

DR. V. SUBRAHMANYAN: Can the author tell us whether the possible potency of a mutant be forecast by studying colony characteristics or by quick microscopic examination?

DR. M. J. THIRUMALACHAR: There is some correlation between high-yielding strains and type of growth character, but this cannot be made use of definitely as a guiding factor.

# Problem of Foam Control in Aerobic Fermentation with Special Reference to Penicillin Production

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**A review of available information on the problem of foam control in antibiotic manufacture is given.**

Adequate control of foaming is one of the major operational problems in submerged aerobic fermentation like penicillin fermentation employing high rates of aeration and agitation under sterile conditions.

Various mechanical devices and chemical methods are in practice to prevent or reduce foaming in industrial processes. Ross<sup>1</sup> has given an excellent general review of the subject. The present discussion is confined to foam control in fermentation of antibiotics in general and of penicillin in particular.

During submerged fermentation of penicillin employing the classical corn-steep lactose medium with high degree of aeration and agitation, an enormous amount of foaming is encountered which must be prevented or controlled in order to save the batch from contamination due to overflow through the propeller-stuffing box of the fermentor and also in order to increase the working volume of the culture. The latter consideration is extremely important from the view point of production economics.

A number of mechanical foam controlling devices incorporated into the design of the fermentor vessels have been described for aerobic fermentations for production of yeast and antibiotics<sup>2-8</sup>. There is no report in literature as to how far these devices have been successfully exploited in antibiotic industries. However, mention may be made of two designs of fermentors which have received considerable attention in penicillin fermentation. Brown and Peterson<sup>9</sup> at Wisconsin used a 30-litre capacity Waldhof-type of fermentor which was first developed at Waldhof-Mannheim, Germany, for yeast production. In this type foam-breaking was effected by a continuous return of the foaming liquid from the top to the bottom of the vessel through a central draft tube. The efficiency of penicillin production was however low unless a chemical anti-foam agent was also added.

The other type described by Chain and coworkers<sup>8</sup> is of considerable interest being a new type of "spargerless" fermentor constructed on the basis of vortex system of aeration by free agitation. The conventional system of aeration is through air spargers in fully baffled fermentor vessels. The spargerless vortex system of fermentors presented no problem of foam control in the sense that foaming reached a steady state and did not overflow out of the vessel. This fermentor has been successfully used for penicillin production and production of bacteria in Prof. Chain's Pilot Plant.

Foam control by chemical antifoams is the usual practice in industrial fermentations producing penicillin and other antibiotics. In penicillin fermentation antifoam agents such as Alkaterge C and Octadecanol are commonly employed mixed with animal, vegetable or mineral oil as carriers for controlling foams. Koffler and coworkers<sup>10</sup> at Wisconsin first reported in 1945 the successful use of 3 per cent octadecanol in lard oil for penicillin production in 80-gal. fermentors. In this factory we have been using 3 per cent octadecanol in groundnut oil. Even the oil alone without adding octadecanol has occasionally been used without any operational difficulty.

Alkaterge C which is a mixture of substituted oxazolines marketed by *Commercial Solvents Corporation*, U.S.A. has received widespread use as an antifoam agent in penicillin industries in America and Europe. A patent on the use of oxazolines as a defoamer was taken in 1948<sup>11</sup>.

In factory practice, empirically determined quantities of these preparations are added at intervals by manual operations or by automatic devices throughout the course of fermentation. These methods are generally considered efficient enough as long as foaming can be controlled to the extent that it does not threaten contamination by overflow, and the concentration of antifoam agent is not toxic to the organism.

It now, however, appears that several other important factors must also be considered in selecting the quality and quantity of antifoam additions. Firstly, the foam inhibiting action should be more or less instantaneous because otherwise, with a slow acting preparation the foaming liquid may overflow by the time the antifoam action sets in. This property is to a great extent dependent on the oil vehicle in which the antifoam agent is dissolved or dispersed. This point will be referred to again later.

Secondly, the quantity of antifoam preparation per addition should be sufficient to effect an almost complete collapse of foam and maintain the defoamed condition for a sufficiently long interval not only for operational convenience but also for better effective aeration. The degree of foam inhibition maintained throughout the course of fermentation is an important consideration because it has been shown that foaming reduces aeration efficiency<sup>8</sup> and may thus affect the yield unfavourably.

Thirdly, metabolism of the vegetable or animal oils in the antifoam preparations may sometimes affect fermentation conditions by altering the pH of medium through liberation of fatty acids and by providing additional carbon source for the fungus. At high aeration rates, lard oil mainly consisting of glycerides of oleic and stearic acids, has actually been found to be utilized by



*Penicillium chrysogenum* as a carbon source in preference to carbohydrates<sup>12</sup>. In fact, lard oil and a number of vegetable oils have been claimed to replace lactose successfully in penicillin production<sup>13-16</sup>.

Several reports have appeared lately indicating stimulation of penicillin production by regulated additions of oils and fatty acids<sup>16, 17, 21-28</sup>. In one report<sup>28</sup> as much as 50 per cent high yield has been claimed by continuous feeding of lard oil starting 20 hr. after inoculation.

A number of commercial surface active agents including mineral oil and silicone antifoam have also been stated to have stimulating effect in penicillin yield<sup>17</sup>. It was found that in respect of oleic acid and lard oil there was a critical concentration above which there was definite inhibition of penicillin production.

Various other synthetic or semi-synthetic antifoam agents for penicillin fermentation have been recently reported in literature. These include a condensation product of ethylene oxide with abietic acid or products containing abietic acid<sup>18</sup>, glycerine ester, ethylene glycol ester and castor oil ester of phenyl acetic acid<sup>19, 20</sup>. These ester preparations are quite novel in the sense that they also supply the precursor of penicillin G.

The above discussion clearly indicates that in penicillin production the role of antifoam agents is much more than merely controlling foams. A systematic investigation in the pilot plant scale seems to be warranted for evaluating various antifoam agents in respect of yield while using a particular strain and medium for fermentation.

During the author's recent sojourn in Prof. Chain's laboratory and pilot plant at the International Training Centre of Microbiology in Rome, work in this respect was already initiated and a simple laboratory method was developed to evaluate quantitatively foam inhibiting actions of various antifoam agents simulating as far as possible the actual fermentation conditions<sup>29</sup>. Concentrations of several antifoam preparations suitable for complete foam control during a period of at least 6 hr. using corn-steep lactose medium have been determined. In this study it was observed that the nature of the dispersing medium had a significant influence on the antifoam activity of octadecanol and Alkaterge C. Alkaterge C dispersed in mineral oil was greatly superior in antifoam action to that in vegetable or animal oil.

Alkaterges in mineral oil have been in use in commercial production of penicillin<sup>1, 30</sup>. Among the advantages claimed are that Alkaterge C in mineral oil did not promote the formation of penicillin K at the expense of penicillin G, it did not turn rancid and most important of all that it was absorbed by the mycelia and did not produce any slime in the fermentation medium<sup>1</sup>.

In the author's experience with a 50-litre fermentor in the pilot plant scale, 0.03 per cent of Alkaterge C together with 0.2 per cent paraffin oil completely inhibited foaming for as long as about 2 days in corn-steep lactose medium using the conventional baffled system of aeration<sup>27</sup>. Dow Chemicals Silicone Antifoam A suitably dispersed was also found to be equally effective at a concentration of 50 parts per million.

Various silicone antifoams are now available commercially. Some of these, the manufacturers claim, have already been tried with success in antibiotic fermentation. In this factory many samples have been received from different manufacturers which are awaiting trials under our fermentation conditions. From the view point of research, the silicone antifoams and also non-lipid antifoam dispersed in mineral oil will be highly useful in studying the effect of fats and oils in penicillin and other antibiotic fermentations.

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### Discussion

DR. S. M. PATEL: Is it necessary to add extra amount of groundnut oil as antifoam in spite of the presence of certain amount of oil in the groundnut cake itself?

DR. D. GHOSH: Yes, extra amount of antifoam is necessary to prevent foaming.

DR. K. S. G. DOSS: In sugar industries we have successfully used a mixture of detergent (aryl alkyl sulphonate) and turkey red oil as antifoams. It may be interesting to try such mixtures in penicillin fermentation.

DR. D. GHOSH: The problem of foam control in penicillin fermentation is slightly different in the sense that the antifoam agent used must be non-toxic to the fungus and also should not give any trouble in extraction and crystallization processes. Any new agent must satisfy these conditions before it could be put to trial.



## Chemical Composition of Mycelium of *Penicillium chrysogenum*

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The chemical composition of mycelium of *Penicillium chrysogenum* Wis. 51-20 (HA. 2) grown in production tanks in a medium consisting of corn-steep liquor, groundnut meal and lactose was determined with a view to finding a use for it. The air-dried mycelium was fractionated successively with isopropyl alcohol, boiling water, sodium hydroxide solution and hydrochloric acid and each fraction further fractionated. Some of the fractions were identified.

The exact chemical composition of the mycelium of *P. chrysogenum* produced in the penicillin factory at Pimpri is of particular interest for three reasons. Firstly, it is available to the order of a few tons every day and we are interested in finding a use for it on the basis of its exact chemical composition. Secondly, the composition of the mycelium is needed for working out the balance sheet in the fermentor between what is added as the media chemicals and what is obtained as end-products. Thirdly, study of the composition of mycelium is of fundamental importance in the chemistry of fungi.

It is known that the chemical composition of fungi varies not only from strain to strain but also according to the way it is cultured and the medium used for the purpose. The complete analysis of any of the fungi has not been carried out so far and we have reports of the isolation of various products by different authors working under differing conditions. As far as *P. chrysogenum* and *P. notatum* are concerned, such reports have been made with various strains using different media and under different conditions. The isolation of the following has been reported.

*Fat fraction*—Fatty oil (m.p. 10-12°C., acid no., 58.8 ; iod. no., 89., and sap. no., 176.6) ; fatty acids after saponification (iod. no., 136.3): myristic, palmitic, stearic, *n*-tetracosanic, oleic, linoleic and elaidic acids ; octadecanol ; a neutral oil with four double bonds ( $C_{36}H_{64}O_4$ ) ; and lipid fraction (acid no., 77.06 ; iod. no., 129)<sup>1-5</sup>.

*Sterols*—Ergosterol, surface culture yielding about 1-3 per cent and submerged culture, 0.1-0.3 per cent<sup>1-16</sup> ; fungisterol<sup>11, 17</sup> ; and stigmasterol<sup>11</sup>.

**Carbohydrates**—Mannitol, erythritol, glycerol<sup>1, 2, 4, 7, 11, 18</sup>; a polysaccharide consisting of glucose, mannose and galactose<sup>18</sup>; chitin, yielding glucosamine hydrochloride on hydrolysis with hydrochloric acid<sup>19</sup>; pentosans<sup>3, 20</sup>; and cellulose<sup>20</sup>.

**Proteins and amino acids**—Crude protein (by Kjeldahl), 30-48 per cent ( $6.25 \times$  Total nitrogen)<sup>1, 2, 9, 19-22</sup>; hot water extracts give lysine, arginine, betaine, and histidine<sup>5</sup>; from the autolysed mould, histidine, arginine, lysine, glutamic acid, aspartic acid, valine, phenylalanine, leucine, alanine, glycine, tyrosine, and proline<sup>19, 22</sup>.

**Vitamins**—Thiamine, riboflavin, pyridoxine, nicotinic acid, biotin and folic acid<sup>9, 21, 23</sup>.

**Phosphorus compounds**—Adenosine triphosphate, mannose-1-phosphate and inositol phosphate<sup>24</sup>.

This paper describes the preliminary work carried out on the composition of the mycelia of *P. chrysogenum* Wis. 51-20 (HA. 2) grown in fermentors in a medium consisting of corn-steep liquor, groundnut cake and lactose at the end of the fermentation cycle.

## EXPERIMENTAL

### Proximate analysis

The fermented broth was collected from the fermentation section at the end of the fermentation cycle, filtered, the mould suspended in water and filtered again. It was then washed with 0.2 N hydrochloric acid to remove traces of calcium carbonate and then finally with water. After washing with a small amount of acetone, the mould was dried at 60°C. overnight. Moisture, ash, crude fat and fibre were determined by the conventional A.O.A.C. methods<sup>25</sup>. The average assay values obtained in batches is as follows: Ash, 5.5; crude protein ( $6.25 \times$  Total nitrogen), 43.7; total lipids, 6.2; crude fibre, 16.1; carbohydrate (by difference), 28 per cent.

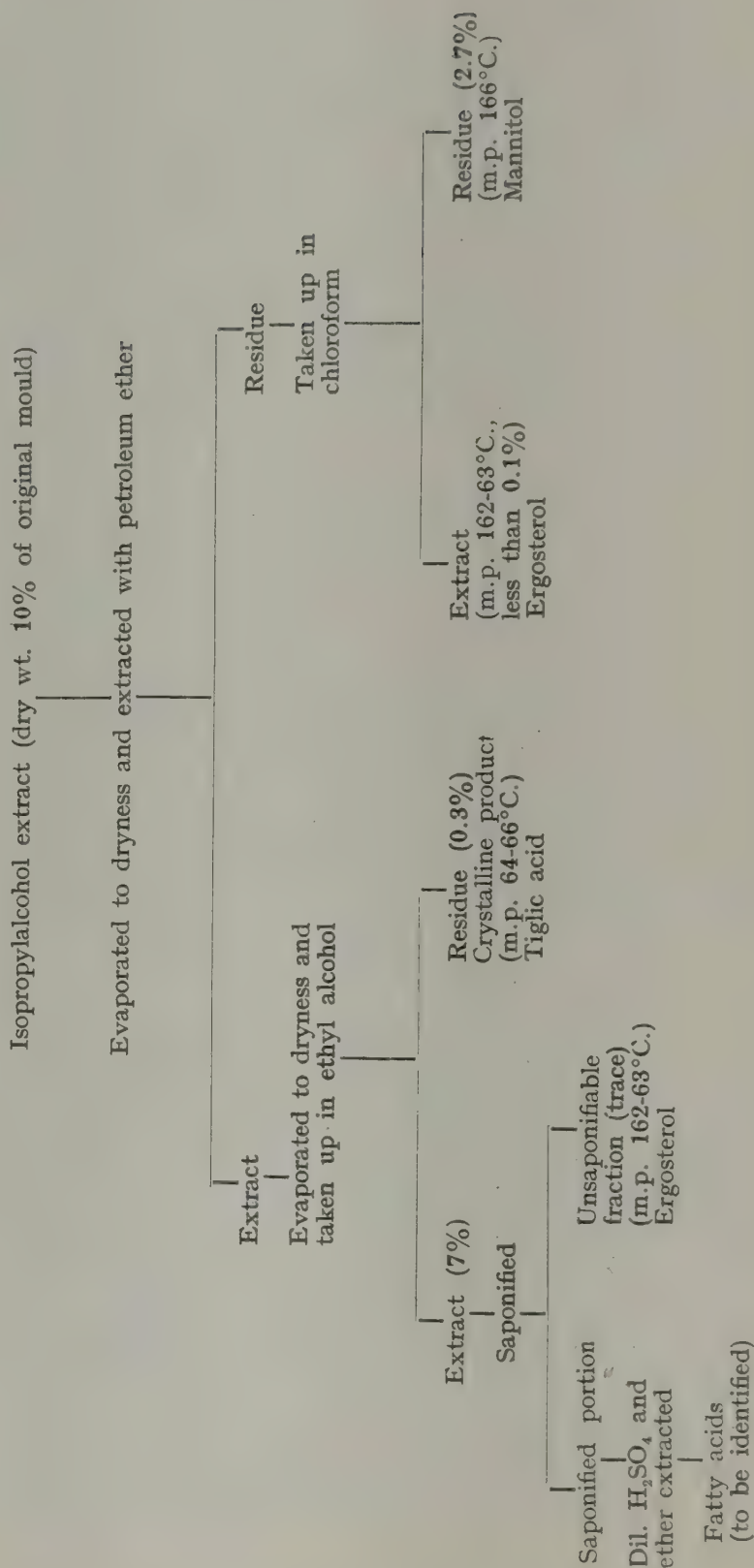
### Isolation of chemical constituents

The fermented broth at the end of the cycle was obtained from the fermentation section and the mould filtered off. It was resuspended in water, filtered, dried in air and then in vacuum to constant weight. The average weight of the dry mould per 100 ml. of the broth in 12 batches was 3.4 g.

The air-dried mould was then fractionated successively with isopropyl alcohol, boiling water, 1 per cent sodium hydroxide, 1 per cent hydrochloric acid, and finally with 12 per cent hydrochloric acid. The solid content of the extracts obtained in each step was as follows: Isopropyl alcohol, 10; boiling water, 28.8; 1 per cent sodium hydroxide, 5.7; 1 per cent hydrochloric acid, 5.8; 12 per cent hydrochloric acid, 22 and residue, 27.7 per cent. Each of the extracts was worked up as follows:

**Isopropyl alcohol extract**—The dry mould was extracted in a Soxhlet with isopropyl alcohol. The extract was evaporated to dryness and worked up as shown in Chart 1.

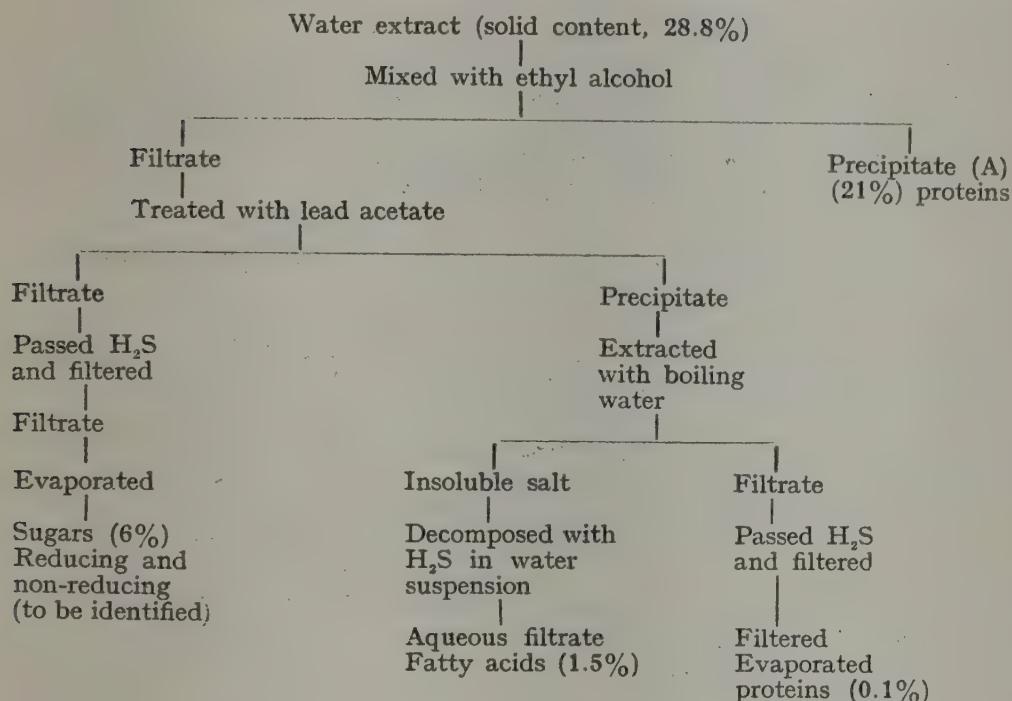
# CHART 1—FRACTIONATION OF ISOPROPYLALCOHOL EXTRACT





*Water extract*—The dried residue after extraction with isopropyl alcohol was boiled with water for 1 hr. and then filtered. The pooled water extracts were worked up as given in Chart 2.

CHART 2—FRACTIONATION OF WATER EXTRACT



The protein fraction A was hydrolysed by 6 N hydrochloric acid for 8 hr. and the hydrolysate subjected to circular paper chromatography and the following amino acids identified: Cystine, arginine, histidine, lysine, serine, glycine, aspartic acid, glutamic acid, proline, alanine, tyrosine, tryptophane, valine, isoleucine and leucine.

*Alkali extract*—The cold 1 per cent sodium hydroxide extract (5.7%) appeared to consist mostly of proteins. It was hydrolysed with 6 N HCl and the hydrolysate subjected to circular paper chromatography. The following amino acids were identified by the Rf values: Cystine, lysine, serine, glycine, aspartic acid, glutamic acid, proline, tyrosine, tryptophane, isoleucine and leucine.

*Dilute hydrochloric acid extract*—This extract appears to contain mostly proteins. The amino acid composition is being studied.

*Twelve per cent hydrochloric acid extract*—The residual mould was boiled with 12 per cent hydrochloric acid for 4 hr. This extracted about 22 per cent of the solids. From this hydrolysate glucosamine hydrochloride was isolated indicating that it arises from the chitins. Part of the hydrolysate was neutralized, and concentrated under reduced pressure. The residue on extraction with hot methanol gave a crystalline material, m.p. 110°C., identified as glucosamine.

The final residue was extracted with hot water. This extract contained hexoses, pentoses, and reducing sugars. They are being identified.

*The final residue*—The ash content of this was 10 per cent (5.5% of the original).

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# Assay of Penicillin with Special Reference to Fermented Broth

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Various methods for the assay of penicillin are critically reviewed from the standpoints of time taken, limits of error, minimum quantities of samples and penicillin units required, chemistry or principles of the process, etc. The chemical assay and microbiological assay methods worked out and adopted to assay the penicillin content of broth samples are described. There is good agreement between the values obtained by the chemical and the microbiological assay methods.

Various methods have been reported in literature for the assay of penicillin, some of which have been reviewed<sup>1</sup>. Each method has its own limitations by way of time required for assay, inherent errors, variations due to interfering substances, etc. The choice of method, therefore, depends mainly on the nature of the sample, the accuracy expected for the assay, and the time by which the assay is required. The assay of any antibiotic can be carried out by two methods, namely, the biological assay and the chemical assay. The ability of an antibiotic to kill or inhibit the growth of living micro-organism is demonstrated in the biological assay method ; it is a direct measure of the activity or potency of the antibiotic. The chemical assay on the other hand makes use of certain chemical properties for the estimation.

The nature of samples that an analyst comes across for the assay of penicillin is given in Table 1. The Table indicates the wide variety of the nature of sample and range of potency, varying from 0.2 u/ml. in body fluids to over 1,00,000 u/ml. in process fluids and from a pure crystalline material to a highly complex body fluid.

In general, the biological methods take about a day for completion and small quantities of samples with as low unitage as 0.02 u/ml. can be handled. This method is not applicable where the results are required within an hour as in the production samples. The chemical methods on the other hand are very fast, easy to carry out but require at least 2,000 units in aggregate for obtaining reliable results and samples with low unitage are not suitable. The foreign substances present affect the values considerably and require elimination for obtaining reliable values. The present work is concerned with the choice of suitable chemical methods to handle the production samples, parti-



TABLE 1—NATURE OF SAMPLES FOR ASSAY OF PENICILLIN

SAMPLES		QTY. AVAILABLE ml.	UNITAGE u/ml.
<i>For Production Purposes</i>		100	100-2,000
Beer (from fermentation)		20	5,000-80,000
Solvents extracts } Aqueous buffer } extracts }	(from fermentation)	10	10,000-4,00,000
Crude crystals } Final crystals }	(from crystallization)	Any quantity	..
<i>For Control Purposes</i>			
Packaged product } Crystalline product }	(for stability and other studies)	Any quantity	..
Blood, serum, } pathological } fluids, urine }	(for experimental animal absorption and excretion studies)	0.1-2.0 50	0.02 10-100
Blood } Urine }	(for clinical studies)	50	0.01-0.02 10-100
Shake flasks (for screening studies)		10-20	200-3,000

cularly the broth, and determination of the reliability as compared with the bioassay values taken as a standard.

### Chemical methods

These can be broadly classified into: hydrolytic, colorimetric and iodimetric methods<sup>2</sup>. The hydrolytic methods are based on the principle that the lactam ring opens up to give a new acidic group which is estimated alkalimetrically. The hydrolysis is carried out by using either penicillinase or alkali. In the former case, pure preparations of the enzyme are required and by carrying out the hydrolysis in bicarbonate solution the method can be made manometric. These methods are applicable to crystalline materials only and are not valid in the case of buffered solutions. Since each ml. of 0.1 N NaOH is equivalent to 59,400 u/ml., fairly large quantities have to be taken for each estimation. Closely similar to these two methods is the hydrogen peroxide method where the acid produced by oxidation is neutralized by alkali and excess alkali titrated. This method is fairly quick but is applicable only to crystalline samples.

Among recent colorimetric methods, particular mention has to be made of the hydroxylamine method of Ford<sup>3</sup> which is based on the principle that hydroxylamine reacts with penicillin to give a hydroxamic acid which gives a complex with ferric ion that can be determined colorimetrically. Since this is not specific for penicillin, as ketones and amides also react with hydroxylamine, a blank is determined by destroying the penicillin with the enzyme penicillinase or even alkali. Different wave-lengths have been reported for the determination of the colour<sup>4</sup>.

Pan<sup>5</sup> has reported the use of arsenomolybdate reagent for the colorimetric estimation of penicillin in broth fluids. The alkali inactivation product of penicillin in neutral solution gives a green colour with the reagent. It is claimed to determine also the penicilloic acid content of the broth samples in presence of penicillin. Yet another method reported is the formation of blue copper complexes with the alkali decomposition products of penicillin<sup>6</sup>. This method, however, has been used only with pure samples of crystalline penicillin salts.

Among the chemical methods of assay of penicillin, the iodimetric method of Alicino<sup>6</sup> has wide applicability from considerations of accuracy, simplicity of operation, time of assay, etc. Considerable work has been carried out to study the factors affecting the reaction<sup>8-10</sup>. Though 9 equivalents of iodine may be needed to react with one molecule of the alkali inactivation product of penicillin, in practice it varies from 8.5 to 9 equivalents of iodine depending upon the particular conditions of the experiment. This factor is determined experimentally for a set of conditions with a working standard of penicillin. The values obtained for this factor with crystalline penicillin G working standard indicate the accuracy of the method. It has been fairly well established that this method yields results with a standard deviation of 1.5 per cent with crystalline samples.

The factor with crystalline penicillin G working standard, in two sets of 7 replicates each and one of 6 replicates, varied from 637-642 (mean deviation, 1.07), 646-654 (mean deviation, 3.3) and 650-654 (mean deviation, 1.1) respectively.

Recently, Vogt<sup>7</sup> has reported a bromimetric method using bromate-bromide mixture for the bromination. Though the author claims the method to be superior to the iodimetric method it does not appear to be so. It is applicable only to crystalline samples, when solutions are prepared freshly. It has been found to vary considerably with slight alterations in conditions, a variation, definitely greater than in the iodimetric method.

### Broth samples

For the estimation of penicillin in broth samples, 25 ml. of broth (for potencies over 1,000 u/ml., i.e. broth samples of 40 hr. age) or 50 ml. (for potencies less than 1,000 u/ml.) is extracted with 50 ml. of amyl acetate at pH 2. An aliquot of the amyl acetate extract (25 ml. or 40 ml. as the case may be) is again extracted with 10 ml. buffer pH 7 and the penicillin in the buffer extract is estimated iodimetrically. Two ml. of the buffer extract is treated with 1N alkali for 15 min., acidified with 2 ml. of 1.1N HCl and kept with 10 ml. iodine (0.01N) for 15 min. and the excess iodine titrated with standard thiosulphate. Simultaneously a blank is carried out by adding 2 drops of hydrochloric acid to 2 ml. of buffer extract, 10 ml. of iodine (0.01N) and titrating immediately with 0.01N thiosulphate.

Besides the experimental error, the value depends on the extraction coefficient, destruction of penicillin at pH 2, etc. Hence, investigations on penicillin recovery are carried out by adding known amounts of penicillin to broth

TABLE 2—RECOVERY STUDY WITH KNOWN AMOUNTS OF PENICILLIN (1870 u/ml.)  
ADDED TO BROTH SOLUTION

FOUND u/ml.	RECOVERY %	CORRECTED VALUE u/ml.	DEVIATION %
1,753	93.6	1,862	-0.4
1,775	94.9	1,886	+0.9
1,717	91.8	1,824	-2.5
1,786	95.5	1,897	+1.4
1,775	94.3	1,886	+0.9
1,763	94.0	1,873	0
1,700	93.6	1,806	-3.5

solution in which the penicillin is destroyed by heating at pH 2 and bringing it back to pH 7. In over 100 samples tested by this method the percentage recovery varied between 92 and 96 with an average of 94 per cent; so a correction factor of 1/16 of the value obtained is usually added. Table 2 shows the results obtained in 7 replicates of a recovery study.

Replicate analyses of a broth sample, 7 replicates (iodimetric method), gave assay between 1,510 and 1,539 u/ml. (mean deviation, 9).

The replicate analyses of an aqueous buffer extract (iodimetric method) of a set of 7 replicates gave assay between 23,420 and 23,680 u/ml. (mean deviation, 90).

Certain precautions are necessary for getting consistent results, such as carrying out the blank determination as quickly as possible and having minimum concentration of iodine in the test solution.

In routine analysis of broth samples of different ages from fermentors and shake flasks, both the iodimetric and biological methods of assay were used. For the biological assay the standard plate method with fish spine beads, using *Bacillus subtilis* as the test organism has been used. Table 3 shows the correlation between the values obtained by these two methods for about 3,000 samples.

It is seen that in over 82 per cent of the samples the correlation is within 10 per cent in the range above 1,000 u/ml. For potencies lower than

TABLE 3—CORRELATION BETWEEN CHEMICAL AND BIOLOGICAL METHODS OF ASSAY

POTENCY	NO. OF SAMPLES	DIFFERENCE (%)			
		Up to 5	6-10	11-20	Over 20
1,000 u/ml.	1900	49	33	15	3
500-1,000 u/ml.	308	43	24	19	14
100-300 u/ml.	—	—	15	15	70



500 u/ml. the iodimetric method gives only a qualitative idea of the presence of penicillin in the broth. In a routine production control, high accuracies in the range are not very necessary.

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# Biological Assay of Antibiotics : Part I— Microbiological Assay of Penicillin

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Three modifications of the agar diffusion method for the biological assay of penicillin using *Staphylococcus aureus*, *S. albus* and *Bacillus subtilis* have been studied for comparison. The various factors which considerably influence the assay, namely, choice of organism, medium and volume of sample poured into the cups, have also been investigated. Of these, the cup-plate technique has been found to be the most satisfactory.

Biological as well as chemical methods for assaying penicillin in the different pharmacopoeial preparations have been described in literature. Chemical methods have proved to be less specific and, therefore, biological methods which have been found to be more specific have been recommended for the standardization of penicillin. In the microbiological methods the potency of a sample of penicillin is determined by comparing its power of inhibiting the growth of a sensitive micro-organism with that of a standard preparation. The standard method for the microbiological assay of penicillin first developed by Abraham *et al.*<sup>1</sup> has been improved upon by various workers<sup>2-7</sup>. Bond<sup>8</sup> has described a modified agar medium containing 0.1 per cent sucrose which has been reported to give well defined zones of inhibition.

The present investigation was undertaken with a view to establishing a suitable method for the routine assay of samples of penicillin. The agar diffusion method was chosen and all the three modifications of this method, namely, the one using cylinders of about 0.5 cm. diam., the one using cups of about 0.5 cm. diam. made in the agar plates and the third one using thick filter paper discs of about  $\frac{1}{2}$  in. diam. were compared. Composition of the medium, volume of sample poured into the cups and the strain of the micro-organisms which considerably influence the range, sensitivity and precision of the method were also studied.

## EXPERIMENTAL

The procedure followed was the same as described by Foster and Woodruff<sup>9</sup>.

### Selection of a technique

*Cup-plate technique*—Tubes containing agar medium were melted in a water bath, cooled to between 45° to 50°C.; a measured amount of a suspension of

suitable micro-organism was added to each tube, mixed well, poured into sterile Petri dishes and allowed to set. Four to five discs of about 0.5 cm. diam, were removed from each dish by means of a borer consisting of a thin walled glass tubing (c. 6 in. long) with a sharp cutting end and having a teat attached to the other end. Dilutions of penicillin, made in sterile phosphate buffer solution (pH 7.0), were filled into cups with Pasteur pipettes. The plates were then incubated for 16 to 24 hr. and the zones of inhibition measured.

*Cylinder-plate technique*—After pouring out the agar medium containing a measured amount of the micro-organism into Petri dishes and allowing it to set, small hollow cylinders of pyrex glass c. 0.5 cm. internal diam. were placed on the agar surface of the prepared plates. The cylinders were warmed slightly before being placed on the agar as suggested by Foster and Woodruff<sup>4</sup> to effect a complete seal. Dilutions of penicillin were filled into the cylinders.

*Filter paper disc technique*—Sterile filter paper discs about  $\frac{1}{2}$  in. diam. were placed on the agar surface and dilutions of penicillin added to the discs immediately.

Comparison of the three techniques was done at the same replicate level on three different occasions. Only one dilution containing 1 u/ml. was used. On statistical analysis the coefficient of variation was found to be the least for the cup and plate technique. Consequently, this technique was adopted for the routine assay of penicillin. The sucrose modified agar as described by Bond<sup>8</sup> and a strain of *Staphylococcus albus* isolated in this Laboratory were used in these experiments.

### Factors influencing assay

The various factors influencing assay—(i) composition of the medium, (ii) test organism used and (iii) volume of the sample filled into the cups—were studied using the cup-plate technique.

TABLE 1—COMPARISON OF THE THREE MODIFICATIONS OF AGAR DIFFUSION METHOD  
(Record of 3 experiments on 3 occasions)

METHOD	AVERAGE ZONE	COEFFICIENT OF VARIATION
	DIAM.* mm.	
Cup-plate	22.16	5.28
	23.75	5.66
	28.95	3.45
Cylinder-plate	20.95	5.49
	23.06	5.42
	25.22	8.87
Filter paper	24.85	9.54
	27.50	7.02
	29.80	8.19

\* Average of 20 zones



TABLE 2—INFLUENCE OF VARIOUS FACTORS ON PENICILLIN ASSAY  
(Record of 3 experiments on 3 occasions)

FACTORS	AV. ZONE DIAM.* mm.	COEFFICIENT OF VARIATION %
1. Composition of medium		
Nutrient agar	29.0, 25.05, 27.35	2.96, 2.74, 2.45
Modified agar with sucrose	28.3, 23.10, 24.60	2.32, 2.79, 3.04
2. Organism used		
<i>S. aureus</i>	19.40, 19.55, 19.80	4.55, 5.37, 5.82
<i>S. albus</i>	18.58, 18.98, 19.80	4.03, 5.64, 2.64
<i>B. subtilis</i>	18.47, 19.18, 17.35	2.56, 2.84, 2.66
3. Vol. of sample		
0.04 ml.	20.20, 18.50, 20.00	8.17, 5.68, 4.65
0.08 ml.	23.25, 23.35, 24.90	5.20, 6.04, 4.50

\* Average of 20 zones

*Composition of the medium*—Nutrient agar and Bond's sucrose modified agar as described were employed to find out the effect of composition of medium on the results of assay.

**NUTRIENT AGAR:** meat extract (derived from 500 g. of meat); peptone, 10 g.; sodium chloride, 5 g.; agar, 30 g.; distilled water, sufficient to make 1,000 ml.; pH of the final medium, adjusted to 7.6 before sterilization.

**MODIFIED AGAR MEDIUM (Bond):** lemco, 3 g.; peptone, 10 g.; yeast extract, 1.5 g.; sodium chloride, 3 g.; agar, 30 g.; sucrose, 1 g.; distilled water, sufficient to make 1,000 ml.; pH of the final medium, adjusted to 7.6 before sterilization.

*Test organisms*—Three organisms, *Staphylococcus aureus*, *Bacillus subtilis* (strains from the Central Research Institute, Kasauli) and *Staphylococcus albus* (strain isolated in this laboratory) were employed in these experiments.

*Volume of test sample*—The cups made in the agar (using 10 cm. Petri dishes and 25 cc. of medium for each plate) were found to take about 0.08 ml. of dilution without overflowing. Two volumes, viz., 0.04 and 0.08 ml. of dilution were tried.

## RESULTS

The results obtained with the three techniques are recorded in Table 1.

The results obtained with the cup-plate method varying with the different factors are given in Table 2. Comparisons were made at the same replicate level on three different occasions.

## DISCUSSION

Various workers have reported that certain factors exert considerable influence on the sensitivity and precision of the biological assay of penicillin. On the basis of the results obtained, these points are briefly discussed below.

*Choice of organism*—According to Foster and Woodruff<sup>2,3</sup> *B. subtilis* is the organism of choice because the zones of inhibition obtained are clear and well-defined against the opaque growth of the bacteria. The zones are, however, smaller than those obtained with *Staphylococcus* spp. The British Pharmacopoeia 1958 has recommended *B. subtilis* as the organism of choice for the biological assay of penicillin. The observations given in Table 2 show that *S. albus* and *S. aureus* give larger zones of inhibition than *B. subtilis*. The zones of inhibition obtained with *B. subtilis* were clearer and had better defined edges than those obtained with *S. aureus* or *S. albus*. *S. albus* gives clearer zones of inhibition than *S. aureus*. A phenomenon resembling Liesegang rings is noticed occasionally while using *S. albus* and *S. aureus* and not with *B. subtilis*.

The results are, therefore, in conformity with the observations made by Foster and Woodruff<sup>3</sup> as also the recommendation made in the British Pharmacopoeia 1953 in respect of the organism used.

*Composition of medium*—Different brands and even different lots of the same brand of meat extract and peptone have been reported to produce marked differences in zone sizes. Variations in concentration of these components have also the same effect. Various workers have used an agar medium of simple composition for the routine assay of penicillin. Bond<sup>8</sup> has reported a modified agar medium with 0.1 per cent sucrose which has been reported to give clearer zones of inhibition. In our investigation comparison was done at equal replicate level on three days using the same batches of nutrient agar and Bond's sucrose modified agar media. The variabilities obtained on statistical analysis have been more or less the same with both the media, although nutrient agar tended to produce larger diameters compared to those obtained with modified agar. It has also been observed that slight variation in the size of zones occurs by using the same batch of medium on different days. The modified agar medium was found to give clearer zones of inhibition as has been reported by Bond<sup>8</sup>.

*Volume of sample poured into the cups*—Previous workers have recommended that the cups (cylinders) should be completely filled avoiding overflowing, as they have observed that small variations in the volume of the sample poured into the cups (cylinders) do not materially affect the results. The results obtained by putting 0.04 ml. and 0.08 ml., into the cups (Table 2) show that the differences in the coefficient of variation are not large between two volumes of the same dilution. Yet, it appears that the larger volume gives bigger zones of inhibition. In order, therefore, to get constant results the volume poured into the cups should be kept constant in all the experiments.

## SUMMARY

Three modifications of the agar diffusion method for the biological assay of

penicillin using *Staphylococcus* spp. and *B. subtilis* have been tried. The cup-plate technique has been found to give the most satisfactory results. The various factors which considerably influence the assay, namely, choice of organism, composition of the medium and volume of sample poured into the cups have been studied. Bond's sucrose modified agar has been found to give well-defined zones of inhibition which are easy to measure. The strain of *S. albus* has been found to be more sensitive than the strain of *S. aureus*. A phenomenon resembling Liesegang rings has been observed with *Staphylococcus* spp. *B. subtilis* has been found to give clearer though smaller zones of inhibition and the edges of zones are better defined with *B. subtilis*. Volume of the sample poured into the cups is not significant provided care is taken to fill the cups, avoiding overflowing and if it is kept constant for a particular dilution for all the cups filled during the experiment.

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# Biological Assay of Antibiotics : Part II—Microbiological Assay of Streptomycin, Aureomycin and Terramycin

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Experimental technique for the microbiological assay of streptomycin, aureomycin and terramycin is described. The media used for streptomycin, aureomycin and terramycin were slightly modified by using 3 per cent agar instead of 1.5 per cent (w/v). The test organism used for streptomycin was *Bacillus subtilis* and for aureomycin and terramycin, *Staphylococcus aureus*.

The cup-plate method was used for the routine microbiological assay of streptomycin (dihydrostreptomycin), aureomycin and terramycin. The experimental technique was essentially the same as that described for penicillin in the previous paper. The compositions of the media and the buffer solution used in making the dilutions of the antibiotics were slightly modified in order to suit the environmental conditions of the laboratory. The strain of *Bacillus subtilis* used for the assay of penicillin was found to be satisfactory for the assay of streptomycin (dihydrostreptomycin) and the strain of *Staphylococcus albus* (of this Laboratory) proved to be sensitive for the biological assay of aureomycin and terramycin.

## EXPERIMENTAL

### Streptomycin

*Composition of the medium*—An agar medium having the following composition was used for preparing the plates: peptone, 6 g.; lemco, 1.5 g.; yeast extract, 3.0 g. ; agar, 30.0 g. ; distilled water, sufficient to make 1,000 ml. The pH of the final medium was adjusted to 7.9 before sterilization ; pH of the medium after sterilization was  $7.9 \pm 0.1$ .

This medium is a modification of the method of Y. H. Loo *et al.*<sup>1</sup> The quantity of agar used is 30 g. instead of 15 g. for 1,000 cc. of medium. This was necessitated by consideration of environment.

*Preparation of plates and filling the cups*—The cups were made in the Petri dishes by using the technique mentioned in an earlier paper<sup>2</sup>. Dilutions of streptomycin were made in potassium dihydrogen phosphate buffer solution of pH 8.0 so as to contain 8, 16, 32 and 64 u/ml. The cups were just filled with these dilutions using the same pipette. The plates were kept in a refrigerator for about 1 hr. to facilitate diffusion of the antibiotic ; thereafter,

the plates were incubated at 31°C. for 16 to 24 hr. In every assay a standard preparation is filled into the cups along with the sample under test. For each assay a graph is drawn to correlate the concentration and zones of inhibition with the standard preparation. The zones of inhibition are measured and the potency of the sample under test calculated with reference to the standard preparation using standard methods. The standard preparations are secondary standards of streptomycin initially standardized for the author by the Central Research Institute, Kasauli.

*Organism*—A standardized spore suspension of *Bacillus subtilis* was used for inoculating the plates. This was found to be the most sensitive of the available organisms to streptomycin.

#### **Aureomycin and terramycin**

*Composition of the medium*—Agar medium having the following composition was used for preparing the plates for the microbiological assay of aureomycin and terramycin: peptone, 6 g.; pancreatic digest of purified casein, 4 g.; yeast extract, 3 g.; lemco, 1.5 g.; agar, 30.0 g.; dextrose, 1 g.; distilled water sufficient to make 1,000 ml. The pH of the final medium was adjusted to 6.5-6.6 before sterilization.

*Preparation of plates and filling the cups*—The plates were prepared by using the same technique as was followed for penicillin and streptomycin. Dilutions were made in 1 per cent solution of potassium dihydrogen phosphate, as has been described in the British Pharmacopoeia 1953 for the microbiological assay of aureomycin in order to contain 5, 10, 15 and 20 u/ml. All further details were the same as described for streptomycin. Incubation temperature was 37°C.

*Organism*—A 24 hr. broth culture of *Staphylococcus albus* (our Laboratory strain) was used for inoculating the plates.

#### **DISCUSSION**

The composition of the medium used for assay of streptomycin has been found to be satisfactory in respect of setting properties. Loo *et al.* have suggested paper-disc technique to be the most satisfactory, while the authors have found the cup-plate technique to be the most suitable for the bioassay of streptomycin. The results were worked out on the lines of the bioassay of penicillin (Part I of this work). The composition of the medium, organism of choice and pH of the medium are kept constant during the experiments. The results thus obtained are satisfactory and reliable.

The method followed for the microbiological assay of aureomycin (chlortetracycline) was as described in the British Pharmacopoeia 1953 and satisfactory results were obtained by this method. *S. albus* (this Laboratory strain) was used and has been found to be sensitive.

Till the publication of 1955 Addendum to the British Pharmacopoeia 1953, no suitable method for the biological assay of terramycin (oxytetracycline) was available in literature. Consequently, the cup-plate assay technique for the routine microbiological assay of terramycin was tried. It has been found that the agar medium for aureomycin and this Laboratory's strain of *S. albus* give



satisfactory results in case of terramycin also. The standard used in this assay was a preparation obtained from M/s *Chas Pfizer Inc.*, New York. The cup-plate technique gave very satisfactory results. It is proposed to try this technique by using the international standard for oxytetracycline as recommended in the Addendum 1955 to the British Pharmacopoeia 1953.

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#### Discussion

DR. P. N. NANDI: In our laboratory we found it difficult to obtain a uniform suspension of *B. subtilis*. I would like to know the method of obtaining a uniform suspension of the organism for the preparation of plates.

The use of 3 per cent agar in the media for the bioassay of streptomycin, aureomycin and terramycin is rather high. We have found no difficulty with 1.5 per cent agar

DR. D. KULKARNI: The spores of *B. subtilis* are fairly stable and there should be no difficulty in obtaining a uniform suspension. I agree with the authors that the zones with *B. subtilis* were better defined and that was the reason this particular organism was recommended in the latest edition of B.P. The need for using higher percentage of agar was due to the brand of agar used.

DR. M. SIRSI: Is it valid to use *B. subtilis*, which was a non-pathogenic organism in the bioassay of penicillin and streptomycin? Will the method designed by the authors for the bioassay of penicillin work in the case of mixtures of penicillins?

DR. J. N. TAYAL: *B. subtilis* (Kasauli strain) had never presented any difficulty to us in respect of obtaining a uniform suspension. As regards the percentage of agar, the higher percentage used in our laboratory was satisfactory because it improved the setting property of the medium; besides, it did not in any way adversely affect the assay.

As to the question of validity of using a non-pathogenic organism for assaying the potency of penicillin, attention should be drawn to the fundamental principle of bioassay, namely, comparison of the test preparation with an international standard preparation in respect to some basic response and statistical analysis of the results and subsequent interpretation. It is, therefore, of no consequence if a non-pathogenic organism was used provided the fundamental principles involved in the bioassay were satisfied. The method of bioassay designed by us was intended to accurately assay the pharmacopoeial preparation of antibiotics from the consumer's point of view. Further investigations are required before the suitability or otherwise of this method for a mixture of penicillins could be commented upon.

DR. K. S. G. DOSS: How would the author explain the phenomenon resembling Liesegang rings?

DR. J. N. TAYAL: It is very difficult indeed to explain. One possibility is the formation of resistant forms of the organism.



# Disc Method for Testing Sensitivity of Antibiotics

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The technique used in preparing discs for antibiotic assay is described. The disc method is easy to perform, eliminates personal factors and gives reasonably reproducible results.

Up till 1953 sensitivity tests were carried out by the cup method. The zone of inhibition with this method depends on the concentration of the antibiotic and the depth to which the cup penetrates the agar. To eliminate errors due to irregular and variable penetration, an instrument was devised in the department (by Major M. D. S. Borcar) that warmed the cups to a particular uniform temperature and dropped them simultaneously in groups of five from a fixed height. The results with this instrument were highly accurate, but the process was time consuming. Hence, in 1954 the disc method was adopted for routine tests. The technique is simple and accurate enough for clinical work.

The discs are prepared in the laboratory. The filter paper required for preparing discs requires selection as the pH of different papers varies from 4.5 to 8.0<sup>1</sup>. Discs of 9 mm. diam. are cut from filter paper (Whatman No. 1), pH 6.8, with a mechanical device, and coloured with various poster colours to indicate different antibiotics, and sterilized in a hot air oven. These colours do not diffuse into the medium, are not inhibitory to any organism and do not affect the potency of antibiotics. Seventy discs absorb one ml. of water; they are soaked in a solution of antibiotic whose concentration is so adjusted that the required quantity of antibiotic is present in a volume of liquid that can be absorbed by 70 discs or its multiples. The discs are dried and stored in a desiccator either at room temperature or at 4°C. Discs of penicillin, streptomycin and aureomycin maintained their full potency for 4 months and those of chloromycetin for 8 months. The penicillin discs lost their potency rapidly and to the maximum extent after this interval but the others were stabilized at a 90 per cent level, which was maintained for a long time. A disc could be used while still wet and gave the same results as the dry ones.

Packed in air tight containers or in cellophane paper envelopes the discs could be sent by ordinary post without any loss of activity during transit.

The quantity of antibiotic in different discs was 4 units of penicillin and 30 ug. of each of the others. The selection of these doses was arbitrary and higher than the concentrations obtained in blood with the administration of the usual dosage ; but it did not affect the merit of the test and gave fairly large and measurable zones of inhibition. Two standard sensitive organisms, *Bacterium coli* and *staphylococcus* spp. were included in every test to evaluate the relative effect of the antibiotics used in the test. The results are expressed in mm. of zones of inhibition as in Table 1.

An antibiotic giving a zone of inhibition of 2 mm. or less is not recommended for therapy if a more effective alternative is available. The number of strains tested in 1953, 1954 and 1955 is given in Table 2 and their sensitivity to different antibiotics in Table 3.

It was observed that in 1953, with the cup technique, more strains had been reported as resistant than in subsequent years with the discs ; but the numbers being small the differences are not significant. During the last three years, there has been no increase of resistant strains of staphylococci, *Bacterium proteus*, diphtheria group and *Neisseria catarrhalis*. The intestinal organisms

TABLE 1—ZONES OF INHIBITIONS WITH VARIOUS ANTIBIOTICS

ORGANISM	ZONE OF INHIBITION, mm.						
	Penicillin	Strepto- mycetin	Chloro- mycetin	Aureo- mycin	Achro- mycin	Ilo- tycin	Terra- mycin
Standard <i>Bacterium coli</i>	0	8	8	3	0	0	2
Standard <i>S. aureus</i>	12	8	9	4	3	9	4
<i>S. haemolyticus</i>	5	9	5	6	1	5	2
<i>S. haemolyticus</i>	0	0	10	8	2	9	3
<i>S. albus</i> , coagulase	11	8	16	6	9	20	10
<i>S. albus</i> , coagulase	3	6	9	4	2	7	3

TABLE 2—NUMBER OF STRAINS TESTED

ORGANISM	YEAR		
	1953	1954	1955
Staphylococci	176	302	527
Streptococci	86	129	188
Colon bacilli	90	50	99
<i>B. pyocyaneus</i>	0	31	81
<i>B. proteus</i>	0	18	12
Diphtheria group	36	38	29
<i>Neisseria catarrhalis</i>	6	29	49
Total	394	597	925

TABLE 3—INCIDENCE OF RESISTANCE OF VARIOUS STRAINS TO DIFFERENT ANTIBIOTICS (%)

	STAPHYLO- COCCI	STREPTO- COCCI	COLON BACILLI	<i>B. pyo- cyaneus</i>	<i>B. proteus</i>	DIPH- THERIA GROUP	<i>N. catar- rhialis</i>
Penicillin							
1953	41	16	86	—	—	16	—
1954	31	6	100	100	100	5	21
1955	29	4	89	100	100	0	10
Streptomycin							
1953	94	6	12	—	—	6	—
1954	0.3	0	4	3	17	3	3
1955	2	2	8	19	25	0	2
Chloromycetin							
1953	2	2	2	—	—	4	—
1954	0	0	0	0	0	0	0
1955	0	0	0	5	0	0	0
Aureomycin							
1953	3	1	34	—	—	0	—
1954	1	0	8	58	95	0	0
1955	2	0	14	72	100	0	2

have shown a gradual increase in the number of strains resistant to orally administered antibiotics. Due to small numbers tested the difference lacks statistical significance and only indicates a trend.

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# Studies on Soluble Enzymes in Penicillin Fermentation Broth : I—Proteolytic Activity

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Proteolytic activity of fermentation broth (Sorensen's formol titration method with casein as substrate) was followed up during penicillin fermentation with *Penicillium chrysogenum* in production tanks. The activity was low in the early stages but increased steadily with the progress of fermentation and tended to reach a maximum towards the end of the cycle. The activity seemed to run parallel with the penicillin titre.

Proteases, the enzymes which catalyse the hydrolysis of peptide bonds are distributed widely in nature. Though the animal proteases have been investigated in great detail and many obtained in crystalline state during the last fifty years, the microbial proteases have been studied only recently. Maschmann<sup>1</sup> studied proteases from a number of aerobic and anaerobic bacteria. An important discovery was made by Linderstrom-Lang and Ottesen<sup>2</sup> when they obtained an extracellular protease from a strain of *Bacillus subtilis* which could transform ovalbumin to plakalbumin<sup>2</sup>. More recently, proteinase from *B. subtilis* has been obtained in pure crystalline form and its action on a number of proteins studied<sup>3</sup>. Damodaran, Govindarajan and Subramanian<sup>4</sup> have studied the proteolytic system of *Bacillus licheniformis*. The enzyme system consisted of a proteinase and one or more peptidases.

Crewther and Lennox<sup>5</sup> obtained a crystalline protease from the culture liquid of *Aspergillus oryzae*. Very few references are available in literature on proteolytic enzyme systems of *Penicillium*. Crude preparations obtained from *P. notatum* have been investigated for proteolytic activity<sup>6-8</sup>.

As in this factory groundnut meal is the main source of nitrogen available to the mould (*P. chrysogenum*) during penicillin fermentation, it was of interest to study the enzyme system responsible for breaking up the groundnut meal proteins into soluble and simpler components which could be utilized for growth and penicillin synthesis.

Japanese workers reported the presence of an enzyme, penicillin amidase, which splits the penicillin molecule at the amide linkage through which the precursor, phenylacetic acid, is connected with the rest of the penicillin molecule. The enzyme had an optimum activity at a temperature range of 25 to 38°C. and pH 7.6 to 8.0. According to these authors flattening of the

penicillin curve towards the end of fermentation was mainly due to the activity of this enzyme<sup>9</sup>. It will also be of interest to find out if such enzymes are present in the proteolytic system of the organism under study and the part played by this enzyme, if any, in the synthesis of the amide linkage.

The present paper deals with the preliminary investigations carried out on the proteolytic activity in mycelia and filtered broth during penicillin fermentation under production conditions in this factory.

## EXPERIMENTAL

### Preparation of samples for analysis

*P. chrysogenum* HA-2 (a selection from the Wisconsin strain 51-20) was grown in sucrose-corn steep medium in seed vessels for 48 hr. and then transferred to large fermentors containing a groundnut meal corn-steep lactose medium. Samples of broth obtained from the seed vessels and production tanks were immediately filtered on a Buchner funnel and the filtrate was used directly for assay of protease activity.

The mycelium was washed with distilled water 6 to 8 times and as far as possible freed from the adhering liquid. An aliquot of the sample was taken for dry weight determination. It may be noted that solid particles of groundnut meal were present in the medium in the earlier stages of fermentation and as such the dry weight determinations were not too accurate.

### Preparation of mycelial extracts

For extraction of enzymes, the mycelium was subjected to the following treatments: (1) freezing and thawing ; (2) dehydration over phosphorus pentoxide in vacuo ; and (3) blenderization with cold acetone in Waring Blender. The activities of the extracts obtained by these methods were comparable but for operational convenience the last method was adopted.

One part of mycelium was blenderized for 1 min. with 10 parts of acetone previously cooled to  $-5^{\circ}$  to  $-10^{\circ}\text{C}$ . and filtered with suction, and the treatment was repeated. Acetone was removed by keeping at room temperature in a draft of air for one hour and finally over phosphorus pentoxide in vacuo.

One part of this powder of mycelium was ground with 3 parts of acid-washed sand at  $0^{\circ}\text{C}$ ., extracted with 20 parts extractant for 30 min. at  $0^{\circ}\text{C}$ ., centrifuged for 15 min. at 1,000 g. (International refrigerated centrifuge, model PR-2). Of the extractants tried, namely, 0.81 per cent sodium chloride, sodium acetate 0.1 M ( $\text{pH}$  5.6), water and sodium phosphate 0.1 M ( $\text{pH}$  7.5), phosphate buffer gave the best results and was therefore used for extraction. Very little activity was obtained in a subsequent extraction of the residual mycelium. The activity in the first extract was therefore taken as the activity in the mycelium. Of course, it is quite likely that all the activity present in the mycelium may not be extracted out by this treatment.

### Determination of protease activity

Protease activity was determined by Sorensen's formol titration method using casein as the substrate. Ten ml. of 2 per cent casein solution, adjusted

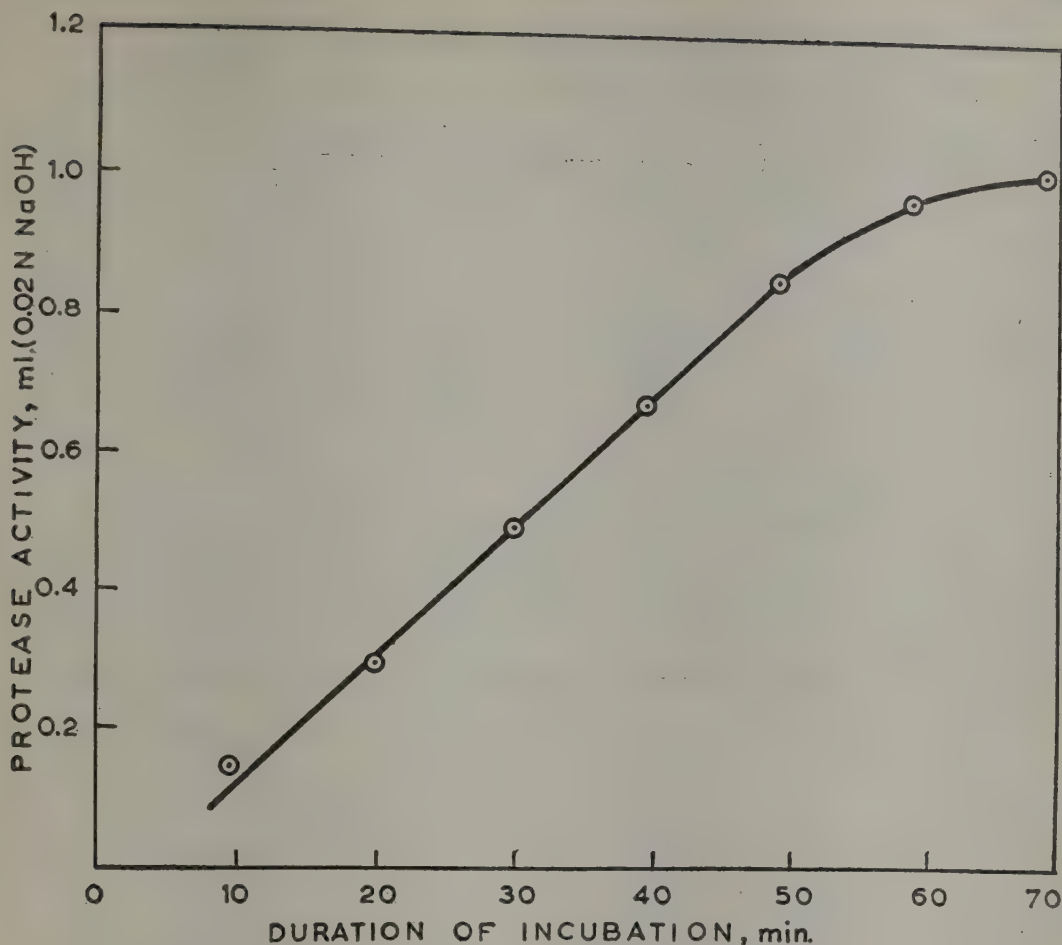


FIG. 1—PROTEASE ACTIVITY AND TIME OF INCUBATION (20 ML. 20% CASEIN, 10 ML. 0.07 M PHOSPHATE BUFFER pH 7.5, INCUBATED AT 30°C. WITH 2 ML. BROTH FILTRATE. TOTAL VOLUME 42 ML., 5 ML., ALIQUOTS REMOVED AT 10 MIN. INTERVAL AND TITRATED AGAINST 0.05 N NaOH).

to pH 7.5, 5 ml. 0.07 M-phosphate buffer, pH 7.5, and broth filtrate or mycelial extract in suitable concentration in a final volume of 21 ml. were incubated at 37°C. After 30 min. incubation, 5 ml. aliquots (in duplicate) were removed and titrated against 0.2N NaOH. Enzyme and substrate blanks were run simultaneously. The results were expressed in terms of ml. 0.02N NaOH. Under the above conditions of estimation there was linear relationship between time of incubation and activity and enzyme concentration and activity if the NaOH titre did not exceed 0.8 ml. (Figs. 1 & 2).

#### Unit of enzyme activity

The unit of protease activity was arbitrarily defined as that amount which catalyzed the hydrolysis of casein to give an increase of 1 ml. in 0.02 N NaOH titre in one hour at 37°C. under the conditions of the test described above. Mycelial dry weight was determined by drying samples overnight at 105°C.

#### RESULTS AND DISCUSSION

The proteolytic activity of the strain of *P. chrysogenum* used in the present investigation is mostly extracellular as *c.* 90 per cent of the activity has



TABLE 1—DISTRIBUTION OF ACTIVITY IN BROTH FILTRATE AND MYCELIUM

DURATION OF FERMENTATION <i>hr.</i>	DRY WT. OF MYCELIUM <i>g.</i>	ACTIVITY, UNITS			ACTIVITY OF BROTH FILTRATE <div>TOTAL ACTIVITY</div> %
		Broth filtrate*	Mycelium†	Total	
24	2.1	244	29.0	273.0	82
32	2.3	428	43.7	471.7	91
56	3.1	570	42.8	612.8	93
72	3.3	580	36.3	616.3	94

\* 100 ml. broth filtrate    † 100 ml. broth

been found to be present in the broth filtrate. The results of a typical experiment are shown in Table 1. The filtrate retained its protease activity on storage for 3 to 4 days at 0° to 50°C. and for weeks in the frozen condition.

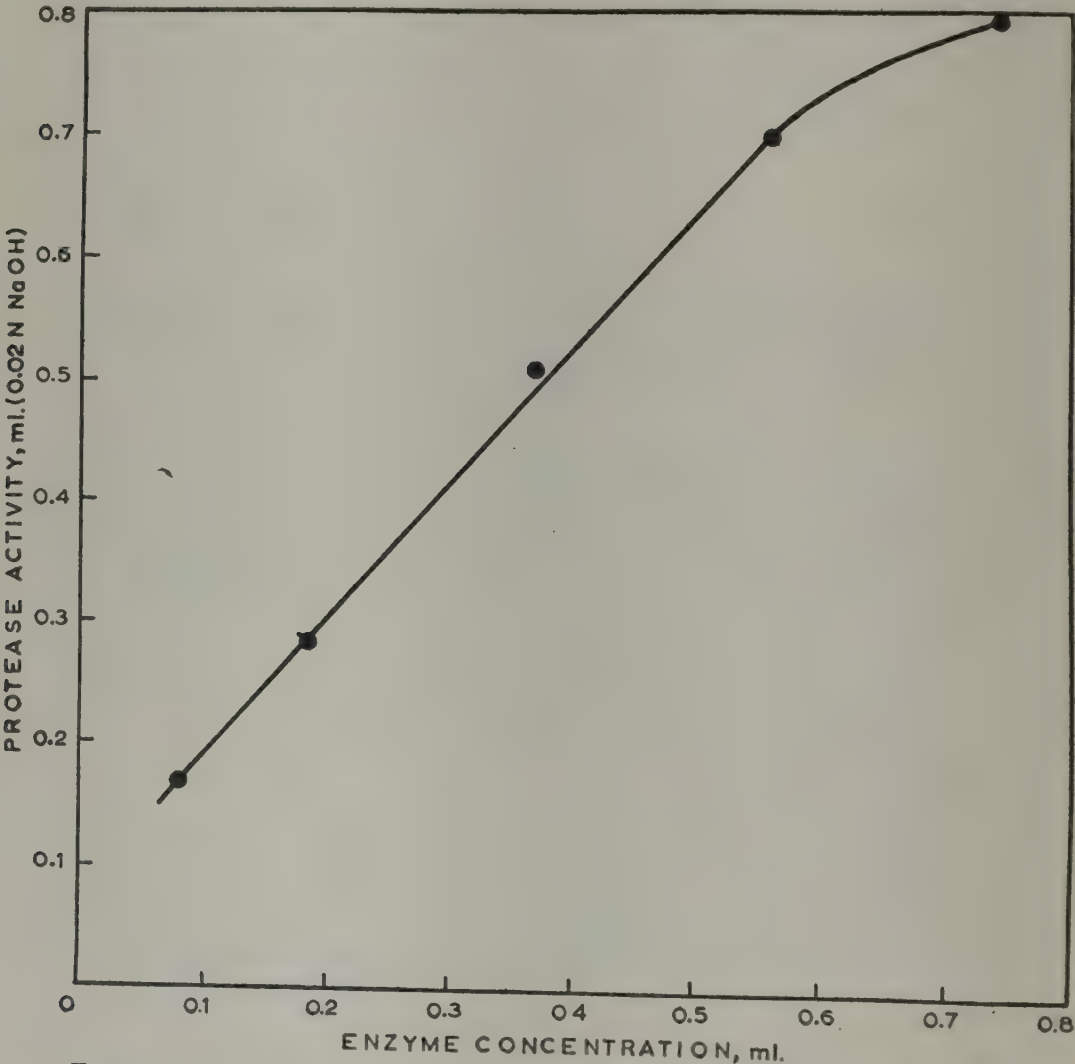


FIG. 2—ENZYME CONCENTRATION AND PROTEASE ACTIVITY (10 ML. 2% CASEIN, 10 ML. 0.05 M PHOSPHATE BUFFER, pH 7.5, INCUBATED WITH BROTH FILTRATE FOR 30 MIN. AT 37°C. TOTAL VOLUME 21 ML.)

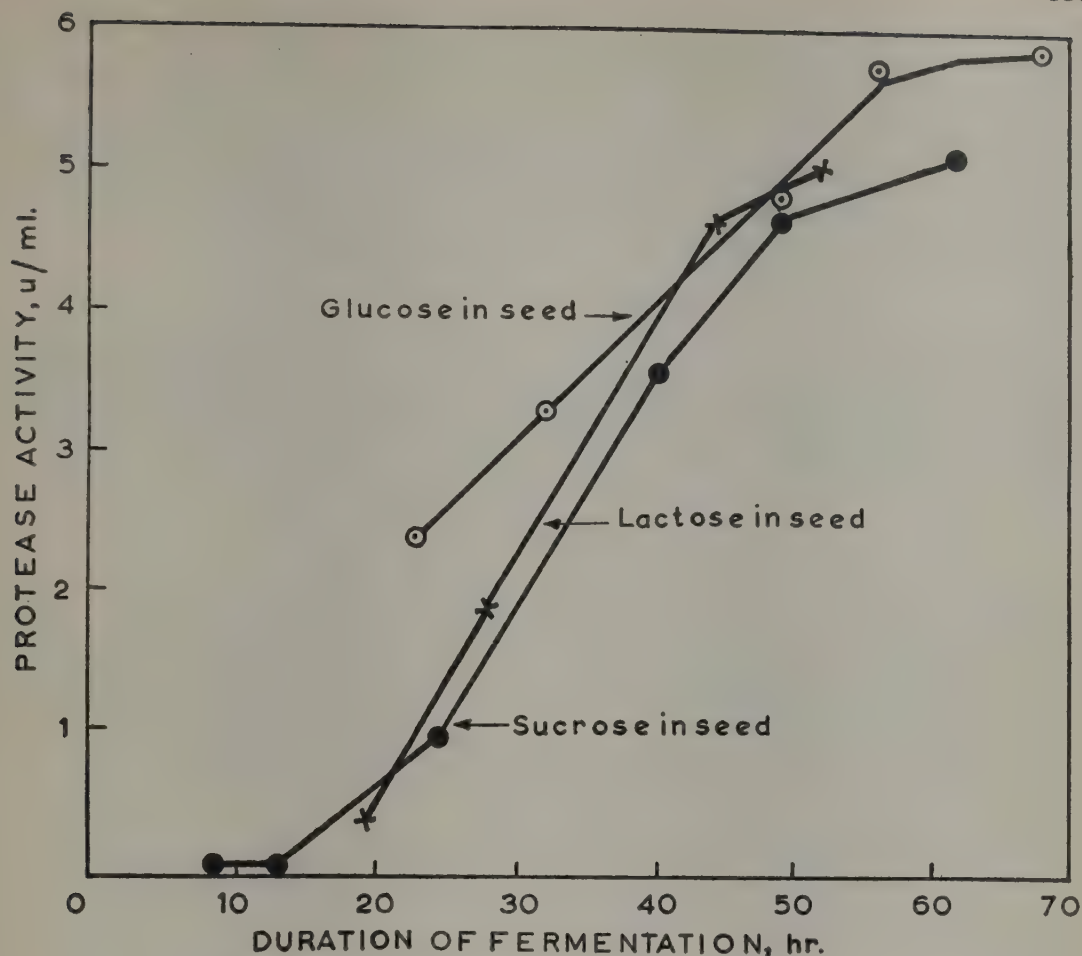


FIG. 3—VARIATION IN PROTEASE ACTIVITY WITH HOURS OF FERMENTATION (10 ML. 2% CASEIN, 10 ML. 0.07 M, PHOSPHATE BUFFER, pH 7.5 AND BROTH FILTRATE INCUBATED FOR 30 MIN. AT 37°C, TOTAL VOLUME 21 ML.)

Prolonged dialysis against distilled water at 0° to 5°C. did not cause any appreciable inactivation. Eighty to ninety per cent of the activity could be precipitated by adding ammonium sulphate to 85 per cent saturation. This precipitation could be repeated without further loss in activity.

#### Variation in protease activity with time of fermentation

Protease activity in the filtered broth as well as mycelium from the seed tank was negligible. In the fermentor, the activity was low in the initial stages, i.e. in the first 14 to 18 hr. of fermentation. This was followed by a sharp rise in activity, the maximum being reached at about 50 hr. after which the curve flattened out. The pattern of this curve followed fairly closely that of penicillin titre. Though there were variations from batch to batch the general trend was the same. Similar results were obtained when glucose or lactose was used as a source of carbohydrate in the seed tank. Results of typical experiments are presented graphically (Fig. 3).

#### Activity on groundnut protein

Proteolytic activity of the broth was also studied using a protein fraction isolated from groundnut meal instead of casein as substrate. The protein was

tritrated in a pestle and mortar with 0.07 M phosphate buffer, pH 7.5 (1 g. in 40 ml. buffer) and heated for 15 min. in a boiling water bath. The pH was adjusted to 7.5 with 1 N NaOH and volume made up to 50 ml. Insoluble material was discarded and supernatant used as the substrate in the usual test system. The proteolytic activity of broth was about half of that obtained with casein as the substrate. This work is being continued.

#### ACKNOWLEDGMENT

Our thanks are due to Shri H. G. Vartak and Shri B. Ganguli for their technical assistance and to the fermentation department for making broth samples available to us.

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#### Discussion

DR. V. SUBRAHMANYAN: You mentioned that during dialysis the protease activity was recovered in the supernatant. Could this protease be then non-protein in nature? It would be interesting to know if any appreciable amount of proteolytic activity is retained by the mycelium and can be made use of as such.

DR. D. GHOSH: The supernatant in question is the soluble portion remaining in the dialysing tube after dialysis. There was very little insoluble material deposited at the bottom of the tube. The question of the protease being non-protein in nature does not, therefore, arise.

SHRI KARTAR SINGH: Regarding the second question, I have already stated that under the condition of extraction method employed only about 10 per cent of the total protease activity of broth suspension was found in the mycelium.



# Studies on Soluble Enzymes in Penicillin Fermentation Broth: II—Invertase Activity

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Invertase activity was determined on dialyzed broth samples using sucrose as substrate and the total reducing sugar formed by Somogyi's method. Invertase in the mould *P. chrysogenum* did not appear to be an 'Endoenzyme' but secreted into the fermentation medium during active fermentation.

The implication of the results in penicillin fermentation has been discussed with reference to the question of adaptation to various sugars during seed and fermentation cycle.

In commercial production of penicillin by submerged fermentation, lactose has all along been the sugar of choice ever since corn steep-lactose medium was first introduced by Moyer and Coghill<sup>1</sup>. The traditional use of lactose in preference to much cheaper sugars like glucose or sucrose has been mainly due to its slow oxidation by the mould so as to maintain a semi-starvation level of supply of carbohydrate, a condition which is considered essential for optimum growth and penicillin synthesis<sup>2</sup>. Recently, Johnson and his group have clearly demonstrated that this near-starvation level of carbohydrate can be accomplished by slow addition of readily oxidizable sugars like glucose or sucrose. Working with both synthetic and corn-steep medium in shake flasks and small fermentors they have been able to replace lactose completely without affecting the penicillin yield by continuously feeding low levels of these sugars<sup>3-5</sup>. From unpublished sources of information it is also gathered that complete replacement of lactose has become commercially feasible by regulated continuous feeding of sucrose.

In this factory the seed is grown in sucrose-corn steep medium before inoculation into large fermentors containing a modified corn steep-lactose medium with partial replacement of corn-steep by groundnut meal.

With a view to replace completely lactose, which is imported, by indigenous cane-sugar in the fermentation medium, a systematic investigation was undertaken to determine the course of sucrose breakdown by *P. chrysogenum* under production conditions. The present paper deals with the invertase activity in mycelium and filtered broth during growth and penicillin production.

The enzyme invertase has been known long since 1828. It has been detected in vertebrates, invertebrates, moulds, yeast, bacteria and other micro-

organisms<sup>7-11</sup>. Yeast invertase has been studied in more detail as compared with invertases in moulds and other microorganisms<sup>12-14</sup>. The presence of invertase has been reported in *P. notatum*<sup>15</sup>. No literature seems to be available on the invertase activity of *P. chrysogenum*.

### EXPERIMENTAL

Samples of broth containing mycelial suspensions were obtained from the seed vessels and large fermentors of the factory at different hours of fermentation and immediately filtered under suction. Invertase activity was determined in dialyzed samples of filtered broth and on extracts of acetone powder of mycelia prepared according to the technique described by Singh and Ghosh<sup>16</sup>.

#### Determination of invertase activity

Invertase activity was determined by measuring the increase in reducing sugar when sucrose was incubated with the source of enzyme. Two ml. of 0.1 M acetate buffer, pH 5.6, 0.5 ml. of 5 per cent sucrose, together with 2.5 ml. of dialyzed sample of filtered broth (1.25 ml. of undiluted broth) dialyzed against distilled water for 48 hr. in the cold or a suitable volume of mycelial extract in a final volume of 5 ml. were incubated at 37°C. Reducing sugar was determined by Somogyi's method<sup>17</sup>, in aliquots removed at zero time and after one hour of incubation. Enzyme and substrate blanks were run simultaneously. When mycelial extracts were used, 1 ml. of 5 per cent zinc sulphate, and 1 ml. of 0.3 N Ba(OH)<sub>2</sub> were added to the test mixture at the end of the reaction. It was then centrifuged and reducing sugar determined in aliquots of centrifugate. In the case of broth, the enzyme action was arrested by Somogyi's copper reagent.

#### Enzyme unit

One unit of enzyme was arbitrarily defined as that amount of enzyme which hydrolyzed sucrose to give one milligram of reducing sugar, expressed as glucose in one hour, under the assay conditions described above.

#### Estimation of inorganic phosphate

Inorganic phosphate was determined by Summer's method<sup>17</sup>.

### RESULTS AND DISCUSSION

Invertase activity was proportional to enzyme concentration within the range of 0.25 to 1.5 ml. of broth in the test system described above. A linear relationship was also obtained between invertase activity and time of incubation (Fig. 1).

Different pH optima have been reported for invertase, depending upon the source of enzyme (yeast, 4.5, vertebrate tissue<sup>6-8</sup>, and *Aspergillus oryzae*, 3.9)<sup>18,19,20</sup>. *Penicillium chrysogenum* invertase has been found to have an optimum pH over the range 5.7 to 6.7 as shown in Fig. 2. In the earlier part of this work citrate-phosphate buffer was used, but it was found that the

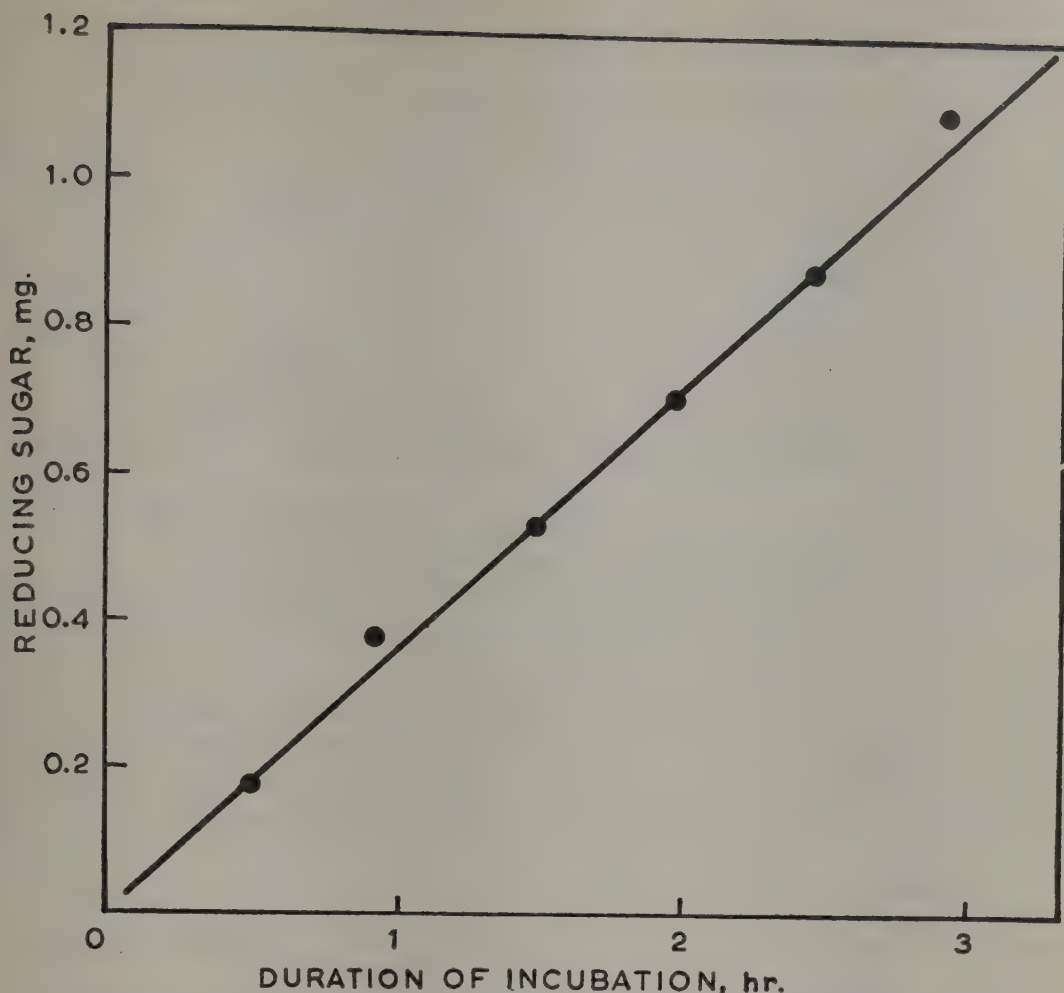
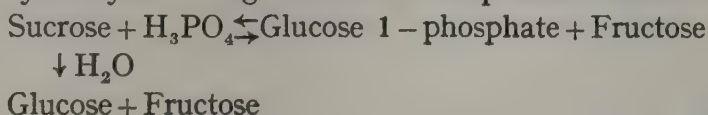


FIG. 1—RATE OF INVERTASE ACTIVITY IN FILTERED BROTH (2.5 ML. OF DIALYZED BROTH, 0.5 ML. OF 5% SUCROSE AND 2.0 ML. OF ACETATE BUFFER, pH 5.6, INCUBATED AT 37°C.)

invertase activity was the same whether acetate or citrate phosphate buffer was used.

Crude invertase preparations from bacteria have been reported to contain sucrose phosphorylase and the existence of competing hydrolytic and phosphorolytic enzyme systems together has been postulated as follows<sup>21,22</sup>:



In the case of enzyme preparations obtained from dialyzed filtered broth of *P. chrysogenum*, the release of reducing sugar from sucrose was entirely due to hydrolytic splitting by invertase because, as can be seen from data presented in Tables 1 and 2, the reaction went as fast in the complete absence of inorganic phosphate and there was no phosphate uptake in the presence of added phosphorus in otherwise phosphate free system.

#### Invertase formation during growth with different sugars in seed medium

The organism was grown in the seed tank with different sources of carbohydrate, e.g., glucose, sucrose, and lactose in 2 per cent concentration. After



TABLE 1—INVERTASE ACTIVITY OF FILTERED BROTH\*  
(72 hr. fermentation)

BUFFER	pH	INVERTASE ACTIVITY u / 100 ml.
Acetate, 0.1 M	5.6	628
Citrate-phosphate, 0.1 M	5.6	632
Citrate-phosphate, 0.1 M	6.3	632

\* The dialyzed enzyme preparation used was free from inorganic phosphorus.

48 hr. of growth in seed tank the contents were transferred to the fermentor containing the usual production-medium containing lactose.

Invertase activity was determined in filtered broth collected from the seed tank as well as from the fermentor throughout the course of seed and fermentation cycle. The results are shown in Table 3.

It will be noticed that in the seed cycle there was no detectable activity in the broth up to 22-24 hr. of growth. When sucrose was used in the medium, a large concentration of invertase was found liberated in the soluble state after 44-48 hr. of growth ; whereas with lactose in the seed medium the activity

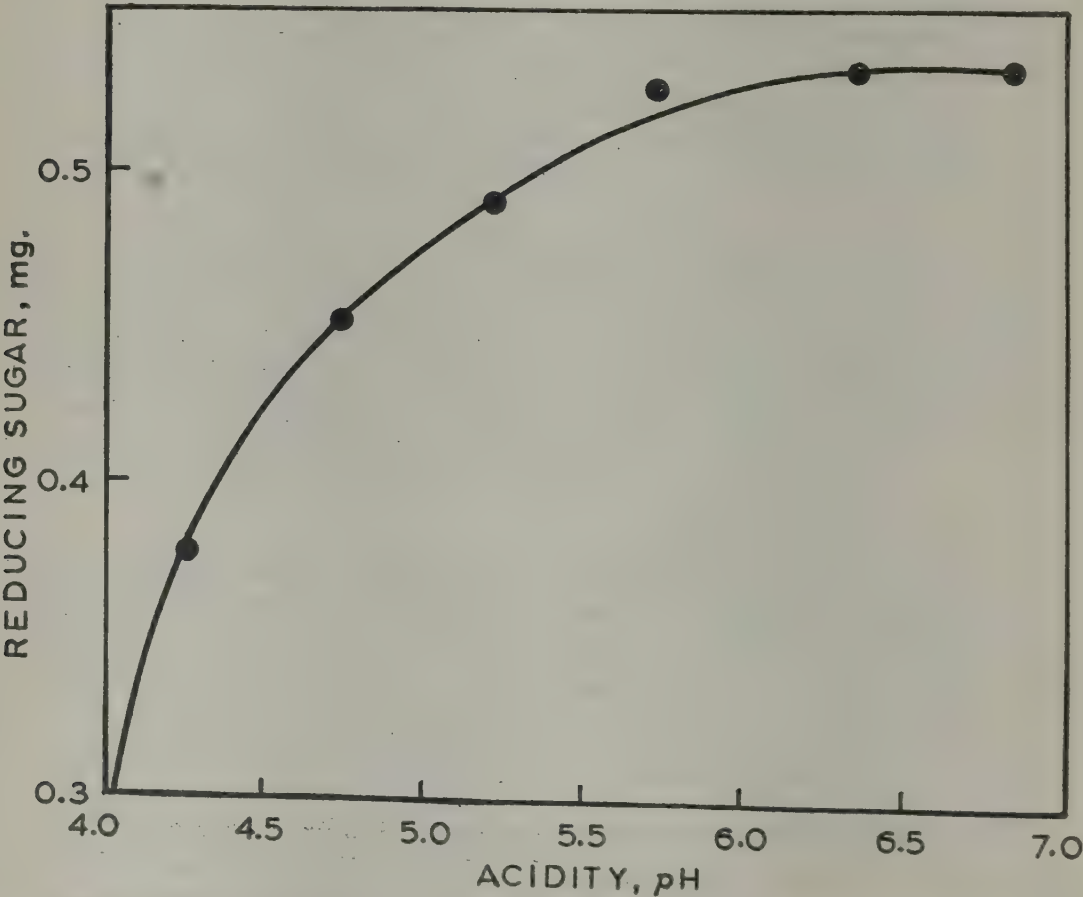


FIG. 2—CURVE SHOWING THE pH OPTIMUM OF INVERTASE IN FILTERED BROTH

TABLE 2—UPTAKE OF INORGANIC PHOSPHATE BY PHOSPHATE-FREE DIALYZED FILTERED BROTH†

SAMPLE No.	DURATION OF FERMENTATION hr.	INORGANIC PHOSPHORUS, mg.		UPTAKE
		Added	Recovered after incubation	
504	32	1.85	1.85	Nil
504	48	1.88	1.89	Nil
528	72	1.76	1.75	Nil

† 2.5 ml. of phosphate-free dialyzed enzyme solution, 2 ml. of 0.1 M acetate buffer pH 5.6, 0.5 ml. of 5% sucrose and 2 ml. of  $\text{KH}_2\text{PO}_4$  containing about 1.8 mg. of inorganic P; final volume 7 ml; incubated for 1 hr. at 37°C.

TABLE 3—INVERTASE ACTIVITY IN FILTERED BROTH DURING SEED AND FERMENTATION CYCLE

SUGAR USED IN SEED TANK	INVERTASE ACTIVITY, u/100 ml.						
	Seed tank broth		Fermentor broth				
	22-24 hr.	44-48 hr.	20-24 hr.	28-32 hr.	44-48 hr.	52-56 hr.	68-72 hr.
Sucrose	0	408	168	456	—	496	552
Glucose	0	40	94	240	304	316	320
Lactose	0	92	88	176	512	552	560

was only about one fourth as much and with glucose the activity was still less, only about 10 per cent.

In the fermentor the lower activity of 20-24 hr. sample with sucrose in seed was apparently due to the effect of dilution. But as the fungus multiplied and as the fermentation progressed, the activity increased to a more or less maximum limit. This trend of increase was also similar when glucose or lactose was used in the seed medium.

The quick induction of invertase synthesis in presence of sucrose in the seed tank indicates that this is an adaptive enzyme in this particular organism. According to the concept of enzymatic adaptation as developed by Stanier<sup>23</sup>, if the dissimilation of a given substance "A" proceeds through a chain of intermediates B, C, D, E, etc. and if the individual steps are under adaptive enzymatic control then growth on medium A will produce cells that are simultaneously adapted to A, B, C, D, E, etc. Growth on C will adapt the cells not only to D, E, etc. but may also adapt the cells to B, and to a lesser degree to A. The low concentration of enzyme when glucose was used in the seed tank can perhaps be explained according to this concept, if we bear in mind that glucose is next to sucrose in the chain of sucrose dissimilation. The same argument can also perhaps be put forward to explain the slower invertase formation in presence of lactose during growth, glucose being an immediate product of lactose breakdown.

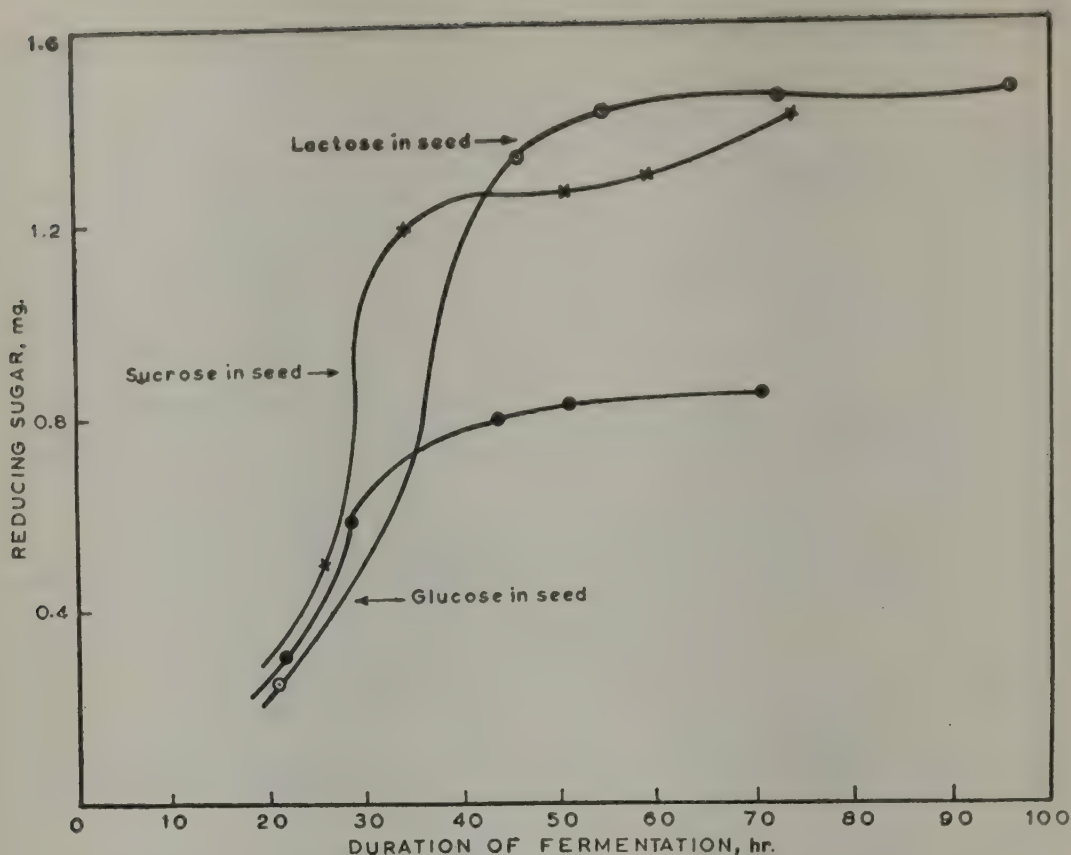


FIG. 3—INVERTASE ACTIVITY IN FERMENTATION BROTH (OBTAINED FROM LARGE FERMENTORS CONTAINING LACTOSE, CORN-STEEP, GROUNDNUT MEAL MEDIUM) AT DIFFERENT HOURS OF FERMENTATION WITH SEEDS GROWN SEPARATELY ON LACTOSE, SUCROSE AND GLUCOSE MEDIUM IN THE SEED TANK

Very recently the formation of invertase in yeast has been reported irrespective of the carbohydrate source used in the culture medium<sup>24,25</sup>.

High concentrations of glucose have been found to inhibit the formation of invertase in yeast<sup>24,25</sup>. We have no sufficient data to postulate that the inhibition of invertase formation in the presence of glucose or lactose is due to a similar effect.

The higher invertase activity of fermentation broth when the cells were grown in lactose compared to that when grown in glucose was perhaps due to thicker growth of lactose grown mycelia in the fermentor (about 5% dry wt. between 50 and 72 hr. of fermentation as compared to about 2.5 to 3% with glucose or sucrose grown seeds).

In all the cases, however, the enzyme titre of the broth ran almost parallel with the trend in penicillin synthesis by this organism. The nature of increase in activity is shown in Fig. 3. In yeast and moulds, the invertase is considered to be an "Endoenzyme" because the enzyme has been found to remain within the cells and not secreted into the culture medium<sup>26</sup>.

The distribution of the enzyme between the mycelium and filtered broth was determined from samples obtained from the seed tank as well as the fermentor. The results are shown in Table 4.

Immediately before transfer of inoculum from the seed tank to the fermentor about 90 per cent of the total activity was obtained in the filtered broth. In



TABLE 4—DISTRIBUTION OF INVERTASE ACTIVITY BETWEEN MYCELIA AND FILTERED BROTH

(Sucrose in seed medium)

SAMPLES FROM	GROWTH hr.	MYCELIAL DRY WT. g./100 ml.	ACTIVITY, u/100 ml.		DISTRIBUTION OF TOTAL ACTIVITY, %	
			Mycelia	Broth	Mycelia	Broth
Seed tank	47	1.27	50	408	10.9	89.1
Fermentor	24	2.1	111	168	39.8	60.2
do.	32	2.3	191	456	29.5	70.5
do.	56	3.1	127	496	20.4	79.6
do.	72	3.3	130	552	19.1	80.9

the fermentor the enzyme was secreted more and more from the cells into the medium with the progress of fermentation. About 80 per cent of the activity was in the broth at the end of fermentation. In *P. chrysogenum*, invertase is not therefore an "Endoenzyme".

## SUMMARY

*P. chrysogenum* has been found to produce the enzyme invertase during growth and production of penicillin in factory conditions. The enzyme is both intracellular and extracellular and is liberated progressively from the cells into the medium as the fermentation proceeds. The pH optimum of this enzyme has been found to be in the range 5.7 to 6.7. Induction of synthesis of this enzyme is greatest when the seed is grown with sucrose as the source of carbohydrate. The invertase titre in the filtered broth of the fermentation medium tends to run parallel with penicillin titre.

## ACKNOWLEDGMENT

The authors are indebted to Dr. K. Ganapathi for his keen interest in the work and to Shri H. G. Vartak and Shri B. N. Ganguli for technical assistance.

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# Production of Enzymes as By-products from Soil Actinomycetes

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One hundred and fifty-five culture filtrates of actinomycetes were examined for their amylolytic and proteolytic activities. Eight strains of these soil organisms which were found to produce the maximum amount of enzymes were studied for the production of amylase and protease.

The extensive distribution of proteolytic enzymes among actinomycetes is indicated by their ability to liquefy gelatine, clot fresh milk and digest albumin, serum and vegetable proteins<sup>1</sup>. Rapid hydrolysis of starch to dextrin and maltose has also been observed in growing cultures of actinomycetes. These enzymatic tests have, in fact, been used in the classification of actinomycetes. In his pioneering work on the taxonomy of soil organisms, Waksman<sup>2</sup> found the proteases and amylases of actinomycetes, in contrast to those of bacteria and fungi, to be highly thermophilic and not influenced by the presence of carbohydrates in the growth medium. In spite of these obvious advantages, no attempts have been made so far to use actinomycetes for the commercial production of amylolytic and proteolytic enzymes.

It was thus of interest as part of a screening programme initiated in the Central Drug Research Institute for the isolation of antibiotically active actinomycetes from Indian soil, to examine culture filtrates routinely assayed for antibiotic activity for their enzymatic activity as well.

## EXPERIMENTAL

The methods of isolation of the actinomycetes have been referred to in another paper<sup>3</sup>. The cultures were grown in liquid media consisting of beef extract, yeast extract, meat papain broth, sodium chloride, ferrous sulphate, potassium phosphate and starch. Incorporation of 0.15 per cent agar was indispensable for obtaining uniformly dispersed growth. The pH of medium was adjusted to 7.2 prior to autoclaving. The tubes after inoculation were incubated in the stationary phase at 28°C. Seven-day old cultures were used in the routine screening.

Choosing eight representative strains from the above cultures, the production of the enzymes was followed up for a period of 15 days.



TABLE 1—NUMBER OF CULTURES SHOWING AMYLASE AND PROTEASE ACTIVITIES

	NO ACTIVITY	LESS THAN 100 UNITS	100-300 UNITS	OVER 300 UNITS
Amylase	25	91	28	11
Protease	26	45	41	12

Units: Amylase—1 unit is equivalent to the amount of enzyme that liberates 100  $\mu$ g. maltose in 60 min at 37°C. from 5 cc. of starch (1%) at pH 7.0.

Protease—1 unit is equivalent to the amount of enzyme that releases 300  $\mu$ g. tyrosine in 60 min. at 37°C. from 5 cc. of casein (1%) at pH 7.0.

TABLE 2—AMYLASE AND PROTEASE ACTIVITIES OF SOME ACTINOMYCETES

CULTURE No.	AMYLASE*	PROTEASE†
181	2.5	245
182	0.8	1330
184	2.7	490
189	1.5	1820
190	0.5	4340
191	1.2	1120
205	10.2	630
207	2.2	1820
212	3.4	3710
222	1.3	2215
230	3.7	0
232	0.0	1470
245	2.1	1470
248	0.3	1125
254	3.7	0
255	2.3	735
272	4.8	105
278	3.2	1820
279	2.2	2485
328	2.7	105

\* mg. maltose formed in 60 min.

†  $\mu$ g. tyrosine liberated in 60 min.

For the assay of enzymatic activity, 1 cc. of the viscous filtrate was incubated with 5 cc. of buffered (pH 7.0) starch or casein (1% w/v) at 37°C. Two cc. of the reaction mixture were withdrawn at 0 and 60 min. and added to 2 cc. of dinitrosalicylic acid reagent for amylase assay and 5 cc. of 7 per cent trichloroacetic acid for protease assay. Maltose formed was estimated by the

colorimetric method of Noelting and Bernfeld<sup>4</sup> and tyrosine in the trichloro-acetic acid filtrates according to the method of Anson<sup>5</sup>.

### RESULTS AND DISCUSSION

A summary of the distribution of amylase and protease in the 155 culture filtrates examined is given in Table 1.

The amylase and protease activities of a few typical strains are given in Table 2.

It would be apparent from Table 2 that there is no correlation between the two enzymatic activities and that the cultures examined provide starting materials for the production of either or both the enzymes according to the different requirements of industry.

Activity of the enzymes reached peak values on the seventh day of incubation and declined sharply thereafter. Incorporation of 2 per cent sucrose in the test medium brought down the activity by 80 per cent. In a medium consisting of wheat bran extract, papain broth and corn-steep liquor the growth was more profuse than in the test medium and activity of the enzyme was comparable. Factors affecting the maximum yield of the enzyme and the standardization of conditions for the precipitation of enzyme are under study.

The results reported in this paper indicate that the actinomycetes isolated are good producers of amylase and protease and may be profitably exploited for the large-scale production of these enzymes.

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### Discussion

DR. S. K. BOSE: Were the  $\alpha$ -amylase and  $\beta$ -amylase activities determined separately?

DR. C. R. KRISHNA MURTI: They were not examined separately, but this examination would be done later on.

# Mycelial Hydrolysate as Medium for Growth of Micro-organisms

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A large quantity of mycelia from penicillin manufacture goes to waste. An attempt has been made to work out an acid hydrolysate which will serve as a medium for growth for micro-organisms. The method of production of this hydrolysate has been standardized. The hydrolysate has been found to be quite satisfactory as nutrient broth or for use in making nutrient agar.

A large quantity of mycelia of *Penicillium chrysogenum* has to be disposed of every day as waste product in penicillin manufacture. It is either composted, used as animal feed or fertilizer after drying or dumped into sea. The mycelia have been shown to consist of about 40 per cent proteins, polysaccharides, sugars and minerals. Acid hydrolysate of meat and of casein have long been found to be suitable routine culture media<sup>1,2</sup>. For more exacting bacteria, or for special purposes such as production of diphtheria toxin or plague vaccine, these hydrolysates have been modified by Mueller<sup>2</sup>, Sokhey<sup>3</sup> and others. It was thought worthwhile to investigate the possibility of using hydrolysates of this mycelium as a culture medium for bacteria. Acid hydrolysate produced in an ordinary autoclave was found to have very good growth promoting qualities for most of the non-exacting bacteria. The method of preparation and the results obtained have been presented here.

## Preparation of the mycelial hydrolysate medium

Four hundred grams of dried mycelial powder were weighed into a 5-litre flask. One hundred and fifty ml. of concentrated hydrochloric acid (commercial) were added to the flask and made up to 3 litres with distilled water. The flask was put in an autoclave and allowed to remain there for 2 hr. after the steam pressure reached 15 lb./sq. in. It was then taken out, allowed to cool to about 60°C. and the contents filtered through cotton. This gave a dark brown liquid with a characteristic odour. The pH was adjusted to 7 using 40 per cent sodium hydroxide solution. About 60 g. of powdered animal charcoal were added to this solution and the solution boiled and filtered through a Buchner funnel. The charcoal treatment removes the dark colour and the characteristic odour. The resulting fluid is pale yellow and has better growth



TABLE 1—GROWTH AND COUNTS OF SOME MICRO-ORGANISMS

ORGANISM	PERIOD OF GROWTH hr.	VIABLE COUNT, ml.	
		Nutrient broth ( $\times 10^8$ )	Mycelial hydrolysate ( $\times 10^8$ )
<i>Bacterium coli</i>	24	8	9
	24	6.7	4.6
	24	4.7	5.1
	48	350	510
<i>Staphylococcus aureus</i>	24	4.2	3.8
	24	5	5.2
<i>Sarcina lutea</i>	24	1.7	0.7

promoting properties. The analysis of the product gave the following percentage composition: Total nitrogen, 257 mg. ; amino-nitrogen, 30 mg. ; sodium chloride, 0.7 mg. Though the quality of mycelium varies slightly from batch to batch, it has been found by trial in over 100 batches in the course of the last 4 months that this proportion remains more or less the same and gives good results.

Various bacteria were grown in this medium and bacterial counts have been made. The total counts were made by turbidimetry method using a photo-electric colorimeter, and the viable count by using the plate method described by Wilson *et al.*<sup>4</sup>, running parallel controls with nutrient broth. Some of the results obtained are given in Table 1.

Colony characteristics and biochemical reactions of the following organisms were studied: *Bacterium coli*, *Bacillus pyocyaneus*, *Bacillus subtilis*, *Sarcina lutea*, *Salmonella typhi* (Army 58), *Salmonella typhi* Ty2, *Salmonella paratyphi*, *Staphylococcus aureus*, *Brucella abortus*, *Brucella melitensis*. The colonies were in all respects similar to those obtained on nutrient agar slants and the biochemical reactions were also similar.

Two per cent agar in mycelial hydrolysate was tried for the bioassay of penicillin by the large plate method using *Bacillus subtilis* as test organism. The zones obtained were quite sharp and distinct ; the bioassay values were reliable and comparable to what we get with beef-extract-peptone agar.

The hydrolysate, diluted 4 times, was used with 2 per cent agar for assaying blood serum penicillin levels using Heatley cups in Petri dishes and *Sarcina lutea* as the test organism. It was possible to detect penicillin levels as low as 0.015 u./ml.

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## Biosynthesis of Penicillin

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From a knowledge of the structure of penicillin and by dissecting the structural elements into various units, it is possible to postulate a scheme for the biosynthesis of penicillin. The pros and cons of the reaction of the components to form penicillin have been discussed leading to the conclusion that penicillin may be synthesized by the mould by the interaction of L-cysteine, phenylacetic acid, dimethylpyruvic acid or dihydroxyisovaleric acid and ammonia. The scheme explains all the facts so far accumulated but the precise mechanisms of condensation and cyclization remain to be elucidated. L-cysteine and dimethylpyruvic acid or dihydroxyisovaleric acid which can lead to L-valine or isoleucine are normally used by the mould for mycelial protein synthesis; when this is slowed down, penicillin appears to be synthesized.

The exact mechanism by which *Penicillium chrysogenum* synthesizes penicillin is not only of theoretical interest but also of very great practical importance. One difficulty confronting us in dealing with this problem arises from the fact that penicillin production by the mould appears to be a very minor and trivial cellular event of practically no vital significance in the economy of the mould. The concentration of penicillin produced by the mould in the fermented broth, under the best of conditions, is only about 0.15 per cent and since even this gets jumbled up with many other metabolites, it is difficult to spot out the mechanisms that are significant from the point of view of the synthesis of penicillin. So, it is not surprising that the earlier work<sup>1</sup> on this problem has given contradictory and confusing results while the method using isotopic tracers, by which it is feasible to trace the chemical pathways of the individual atoms in the molecules, is the only one that has yielded definite and promising results.

The biosynthesis of penicillin involving the mould, its metabolism and the media chemicals, is a multidimensional problem and has to be dealt with at the mycological, biochemical and chemical levels. The three important questions that require answers in this connection relate to the elucidation of: (i) The factors of mycological nature involved in the biosynthesis of penicillin by the mould; (ii) the biochemical mechanisms and the specific enzyme systems involved and the optimum conditions for their functioning; and (iii) the chemical pathways leading to the synthesis of penicillin starting from the elementary media chemicals or constituents.

These questions have attracted the attention of many workers<sup>1,11</sup> and consi-



derable data have been published. An attempt has been made to marshal the data available and to have a coherent picture which will explain all the facts known and indicate further avenues of work on realistic lines.

As a result of studies on penicillin fermentation carried out under various conditions in shake flasks, small fermentors and even production runs, the following conditions have been found to be associated with good penicillin production (though the cause-effect correlation between them and the penicillin yield cannot be vouchsafed<sup>2-5</sup>): (i) The mycelium should not be actively multiplying or should be undergoing only slow development; (ii) the sugar should be fed at a "starvation level"; (iii) sufficient amount of dissolved oxygen in the medium should be available for the cells; it is believed that the degree of aeration which is employed to cause this oxygen solubility always limits the quantity of penicillin produced; (iv) carbon dioxide should probably be available, (0.25 per cent being the optimum, and 1.0 per cent being toxic<sup>5</sup>) thus showing that there is some carbon dioxide fixation involved in penicillin synthesis; (v) assimilation of ammonia appears to be a prerequisite for high penicillin yields<sup>5</sup>; (vi) the pH of the fermenting broth should be round about 7.0-7.6 and (vii) the temperature of fermentation should be 24° to 28°C.<sup>3,4</sup> (20°C., according to a recent publication); a temperature of 32°C. has been reported to be injurious<sup>4</sup>. It is not known how far the above conditions actually govern penicillin production. In addition, these give no indications about the actual mechanisms involved or the exact part they play in penicillin biosynthesis. A number of reports are available which give the materials or the physical conditions claimed to enhance penicillin production, such as some of the amino acids, acetate, antifoam, etc. but it is not known how much importance has to be attached to them, because either the experimental conditions are not well controlled, or the yields involved are very low compared with what is obtained these days.

The knowledge accumulated so far indicates that for the biosynthesis of penicillin, the presence of the mould is essential. Penicillin appears to be synthesized within the cell and excreted out into the medium. Experience in the plant indicates that the type and the physiological state of the mycelium obtained and the nature of metabolism going on govern the degree of penicillin synthesis. Though penicillin synthesis is an intracellular process, it does not mean that penicillin synthesis cannot be carried out by cell free filtrates which contain all the required enzymatic systems. This is an interesting problem that has to be studied.

*Penicillium notatum* and *P. chrysogenum* have the characteristic property of producing penicillin. Only a very few strains are known to produce no penicillin. But this penicillin producing property is not confined only to this group. It has been found<sup>6,7</sup> that 25 penicillia, 7 aspergilli and the totally different *Trichophyton mentagrophytes*, *Malbranchea pulchella* all produce penicillins. There may even be additions to this list with further investigations. Study of the mechanism of penicillin production by these moulds which are able to use different media constituents and under different conditions would be of great interest since it may throw some additional light on the biosynthesis of



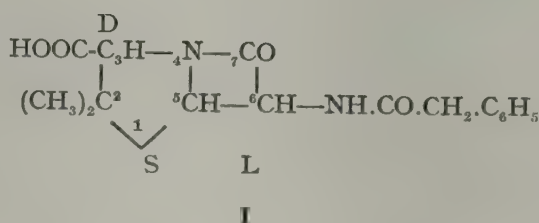
penicillin. The other interesting feature is that a number of penicillins with different side chains are produced by the above mentioned moulds. So far, seven penicillins have been definitely characterized.

*P. chrysogenum* has also the unique property of utilizing a number of compounds of formula  $R.CH_2.COOH$  and forming artificial penicillins with corresponding side chains. A number of such artificially synthesized penicillins are known<sup>9-12</sup>; of these, penicillin O is in actual use and penicillin V is just coming into prominence for oral therapy. So, it is probable that by utilizing the acids available in the metabolic fluids, more penicillins with other side chains may be synthesized by the moulds and these could be isolated and tested therapeutically.

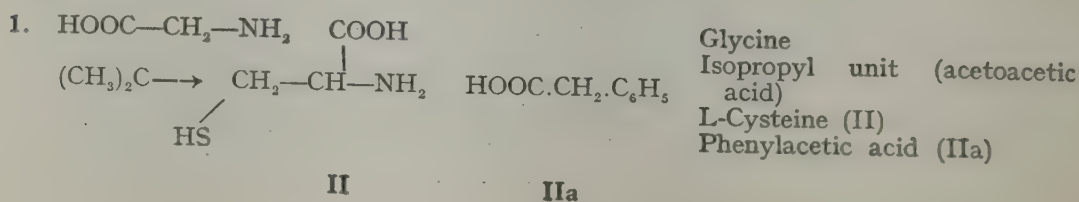
The penicillin producing capacity, from the quantitative point of view, is strain specific. Each strain has a maximum penicillin producing capacity and the optimum conditions for these need not be the same for all strains; in fact they vary from strain to strain. The wild strains occurring in nature are all poor penicillin producers judging from the standard of performance of the strains now used in industry. The high yielding strains are all artificially evolved by mutations involving irradiation with X-rays, ultraviolet rays, nitrogen mustard treatment and selection. The monumental work of Backus<sup>13</sup> has resulted in the development of very superior strains.

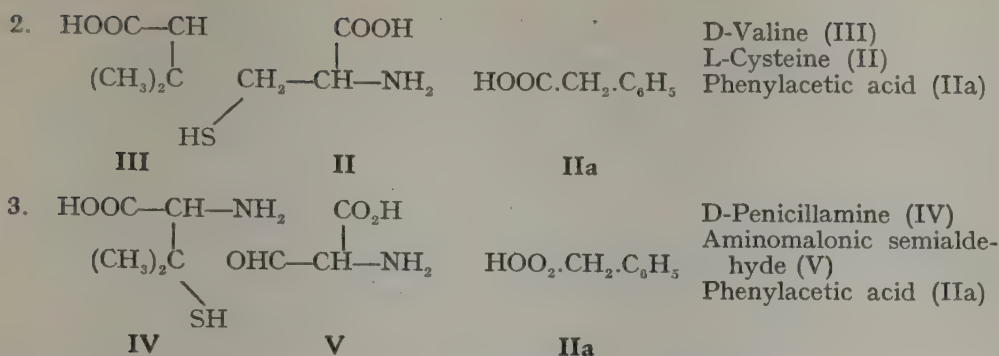
Work prosecuted on the basis of chemical reasoning has provided with some significant results, though the picture as a whole has not been completely elucidated. Since the structure of penicillin is now known, it is natural to dissect the structural elements into various units and to speculate, on the basis of the current biochemical knowledge, how they are likely to arise and then combine to yield penicillin. Such a speculation has the merit that it offers some starting points for the attack of the problem.

Penicillin (I) is an extraordinary type of molecule. There are two asymmetric centres in it, the carbon atom number 3 having the D, and that numbered 6 having the L configuration. The two rings are *cis* locked, and the molecule is semi-circular.



The molecule could be dissected into the following components in three ways as follows:

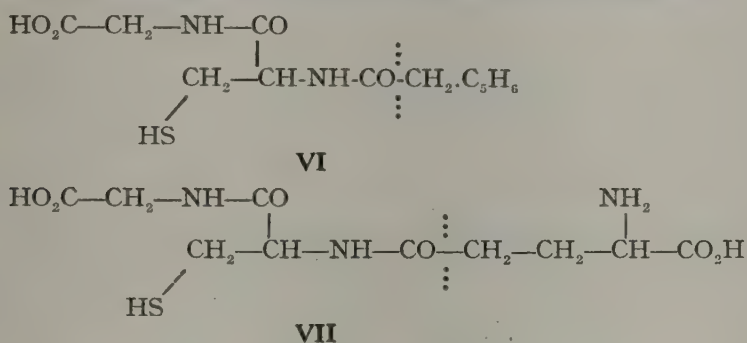




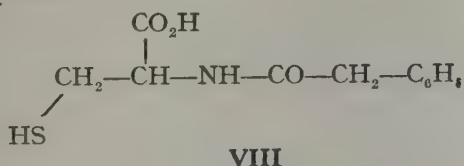
D-Valine (III)  
 L-Cysteine (II)  
 Phenylacetic acid (IIa)  
 D-Penicillamine (IV)  
 Aminomalonic semialde-  
 hyde (V)  
 Phenylacetic acid (IIa)

It has to be seen whether the biosynthesis in these three ways can be supported with experimental facts.

If the penicillin unit is taken omitting the isopropyl residue ( $\text{C}_2$ ) (VI), a formal resemblance to glutathione (VII) can be detected as follows:



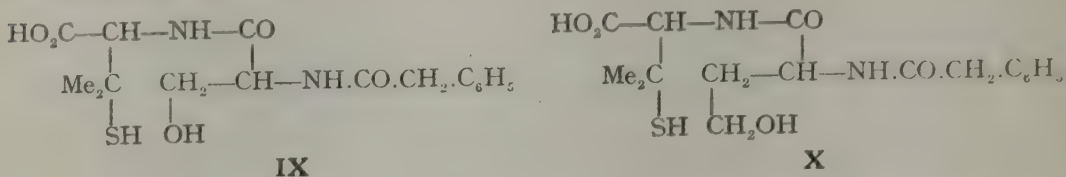
The biosynthesis of glutathione has been assumed to proceed by the condensation of cysteine and glutamic acid and this peptide then undergoing further condensation with glycine, with the two peptide bonds being formed by the mediation of adenosine triphosphate (ATP)<sup>14</sup>. In analogy with this, it can be postulated that the condensation of phenylacetic acid with cysteine yields peptide (VIII) which on condensing with glycine can yield the tripeptide (VI). This on condensation with acetoacetic acid and cyclization can give rise to penicillin. Or the peptide (VIII) can condense with D-valine and undergo cyclization to furnish penicillin.



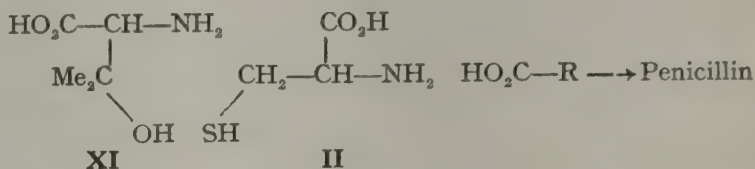
Results with the isotopic work have shown that this is not the case. Though glycine is incorporated into the penicillin molecule, the methylene carbon atom is incorporated at the carbon atom number 6 of penicillin and not at 3. D-valine also is not used in such a way. Hence this scheme does not represent what is going on in the mould.

The idea that penicillamine (IV) can be a precursor must have taken root as a result of the facts that it is related to cysteine, that it was the first amino acid degradation product of penicillin to be isolated, and that it has been used to effect the synthesis of penicillin under the biogenetic conditions<sup>11</sup>. But the work carried out with the isotopic compounds shows that penicillamine does

not serve as a precursor for the biosynthesis of penicillin. Two more penicillamine derivatives, (IX) and (X), which have a very close structural and formal resemblance to penicillin, have also been shown not to serve as penicillin precursors<sup>15,16</sup>.



Hockenhull, Ramachandran and Walker<sup>17</sup>, as a result of their observations on the biosynthesis of dimethylpyruvic acid in *Aspergillus niger*, suggested without experimental proof, that the biosynthesis of penicillin may proceed by the condensation of D-β-hydroxyvaline (XI), L-cysteine (II) and precursor acid.

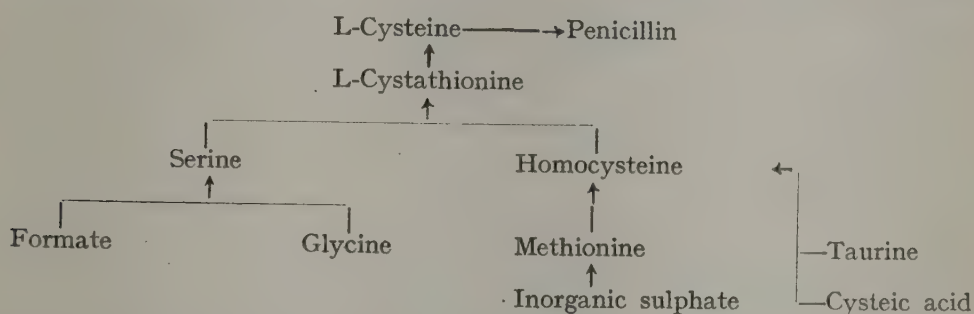


In contrast to the above, the work done using compounds with the isotopically labelled atoms and studying their incorporation into the penicillin molecule, has yielded definite results though all the details have not yet been unravelled. By using sulphate, containing radioactive sulphur, it has been shown<sup>19</sup> that it is incorporated into the penicillin molecule. In fact, in the manufacture of penicillin, inorganic sulphate is added to the medium as a source of sulphur. It has also been shown<sup>21</sup> that about 30 per cent of the added sulphate is converted into methionine, and that L-methionine and L-cysteine are incorporated into the penicillin molecule in preference to sulphate. The other compounds shown to be utilized are taurine and cysteic acid<sup>20</sup> but those more effectively utilized are homocysteine, cystathionine, methionine and L-cysteine<sup>20</sup>. Formate has also been claimed to be incorporated, though the exact position of this has not been determined<sup>22</sup>. It is very likely that it is used in the synthesis of serine from glycine as is shown later on.

The most outstanding piece of work has been reported by Arnstein and Grant<sup>23,24</sup> who have established a number of significant facts. They studied in small scale microfermentations, using synthetic media, the incorporation into the penicillin molecule of the added amino acids, glycine, serine, valine and cystine labelled in different positions. After adding these singly into the media, the penicillin was isolated after fermentation, and the position of the labelled atom was determined by degradation experiments. Glycine and serine were incorporated into penicillin but much less readily than L-cystine. Glycine gets into the β-lactam part of the molecule, the α-carbon getting into the C<sub>6</sub> position chiefly. The conversion of glycine into serine by the mould was proved by the fact that, after the addition of radioactive glycine to the medium, radioactivity was detected in the serine of the mycelium protein. Serine also gets incorporated into the β-lactam portion, which shows that it is also converted

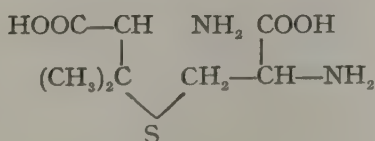


into L-cysteine obviously *via* cystathionine. L-cystine was the one, incorporated intact into the  $\beta$ -lactam portion (atoms 1, 5, 6, 7) with minimum dilutions and this was shown beyond doubt by using the triple labelled ( $\beta$ -C<sup>14</sup>, S<sup>35</sup>, N<sup>15</sup>) cystine. L-cystine was incorporated five times more effectively than the D-isomer, the latter appears to get converted into the L-isomer before it is utilized. It is clear that glycine and serine serve as the precursors of L-cysteine and the usual type of synthesis of L-cysteine, as has been established for other organisms, appears to be going on in *P. chrysogenum* also as follows:



The fate of the amino acids added was found to be as follows: (i) about 1 to 3 per cent of these was incorporated into penicillin, (ii) 20 to 50 times the radioactivity as that in penicillin was detected in the mycelial protein thus indicating that the major course of reaction with these amino acids is the synthesis of protein, and (iii) the acids were also extensively metabolized into respiratory carbon dioxide. This explains why the earlier workers did not obtain consistent results, because they might not have allowed for this extensive metabolism of the compounds added.

Regarding the other component, some specific clues have been obtained but the picture is far from complete. It has been reported<sup>25</sup> that acetate is incorporated much more readily than lactate<sup>22</sup>, but the exact position has not been found out. This probably confirms the claim that acetate stimulates the production of penicillin<sup>26</sup> and the incorporation of acetate in the synthetic medium has a scientific basis. There are a number of ways in which acetate, which is a very versatile biosynthetic unit, can take part in the reactions. Stevens *et al.*<sup>27</sup> found that the carboxyl group of valine is incorporated into the carboxyl of the penicillin molecule. Arnstein and Clubb<sup>28</sup> using the double labelled (C<sup>14</sup>, N<sup>15</sup>) valine have established that while the carbon skeleton of valine is incorporated intact into the penicillamine portion, the nitrogen atom of valine is not. In support of this, it was also found that there is no striking difference between the incorporation of DL- and L-valine. Evidently, valine as such is not the precursor but the deaminated product, which may be dimethylpyruvic acid or dihydroxyisovaleric acid. It is also of interest to know

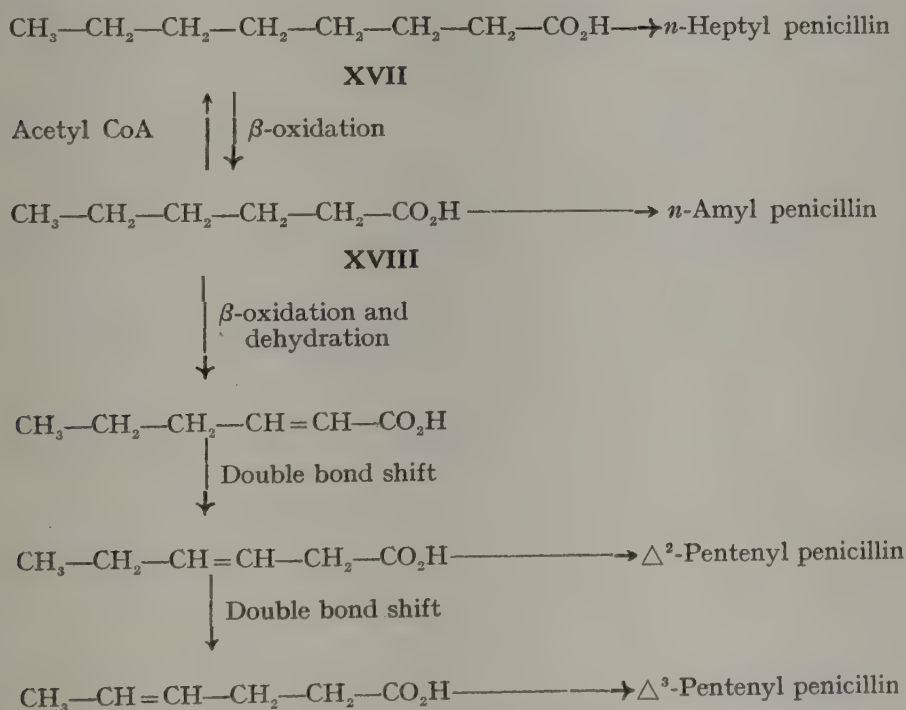


XII



been found<sup>32,33</sup> to be incorporated to the extent of about 93 per cent into penicillin. It was also observed that the respiratory carbon dioxide contained radioactivity<sup>32</sup>. Phenylacetic acid arises from phenylalanine by well-known reactions by oxidative deamination leading first to phenylpyruvic acid which gets further oxidised to phenylacetic acids, or phenylalanine also gets decarboxylated to phenylethylamine (actually isolated from corn-steep liquor) which further gets converted into phenylacetic acid. Since the quantity required for penicillin synthesis by the superior strains cannot be supplied from the media constituents or by the synthetic activity of the mould, it has to be added to the medium. As with the amino acids, a great deal of the acid added is oxidised by the mould, and only a small portion is utilized for penicillin synthesis.

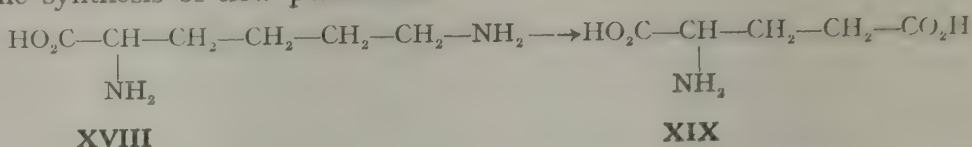
Many other acids are also used by *P. chrysogenum* in the production of the side chain of penicillin. Tyrosine gives rise to *p*-hydroxyphenylacetic acid, and this leads to the biosynthesis of penicillin X. The aliphatic side chains all arise from the fatty acid metabolism of the mould involving either the synthesis of the fatty chains or the degradation of long chain fatty acids. Two of the fatty acids involved, significantly enough, are the C<sub>6</sub> (caproic, XVII) and C<sub>8</sub> (caprylic, XVIII) acids. It is also significant that no C<sub>7</sub> acid side chain has been isolated. The metabolism of these acids involves the beta oxidation of the carbon chain; these acids are formed with an even number of carbon atoms and with unsaturation. The acids involved and how they arise are indicated below:



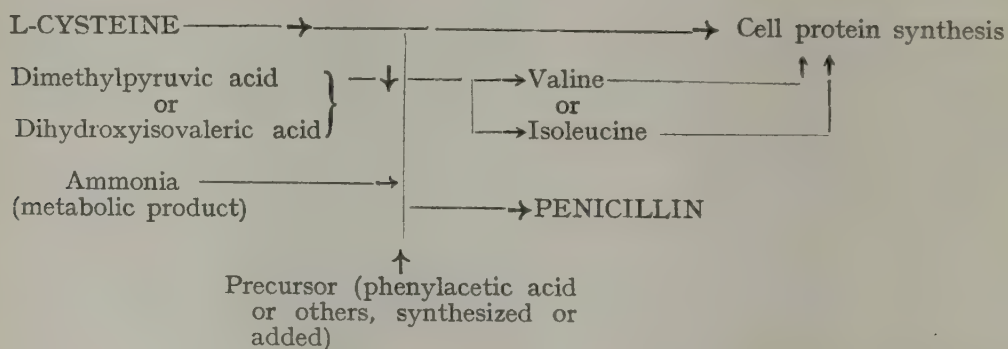
The other interesting penicillin isolated is synnematin B also known as cephalosporin N<sup>8</sup>, which contains  $\alpha$ -amino adipic acid (XIX) side chain. This acid evidently arises from lysine (XVIII)<sup>34</sup> and some reports state that this acid (XIX) serves as a precursor for lysine in *Neurospora*<sup>35</sup>. This amino acid (XIX) is also found in the protein of corn<sup>36</sup>. It is of interest to meet with



such a side chain which indicates that the moulds may be utilizing similar acids for the synthesis of new penicillins.



In the light of the results reported so far, and the other observations made in the strain selection work, some type of a picture of the biochemistry of penicillin synthesis can be had. The work of Backus and Stauffer<sup>13</sup> which has resulted in the evolution of strains with superior penicillin producing properties, involves 12 to 14 steps from the wild strain and in these steps, treatment with X-rays, ultraviolet rays, nitrogen mustard and selections are involved. In each stage there is probably some genetic mutation involved. Backus and Stauffer<sup>13</sup> in describing their selection work observe: "progressive increase in ability to produce antibiotics, at least as measured by test conditions, has been paralleled by a more or less progressive reduction in vegetative and reproductive vigour. Strains rated as highest are all extremely degenerative and weaklings as compared to the wild type strains. It is unlikely that these relationships are accidental or meaningless." If the reduction in vegetative or reproductive vigour is taken as indicative of the reduced rate of cell material synthesis and if it is assumed that penicillin production starts when the mycelium has almost stopped multiplying, it can be reasonably concluded that reactions associated with cell material synthesis stop and give place to penicillin synthesis, or the compounds that usually go in for the synthesis of the cell materials are channelled out for the synthesis of penicillin. The two need not be exclusive of each other. If the latter be correct, then, at least some of the compounds serving as precursors for penicillin synthesis should be those being used for the synthesis of cellular products. This probably appears to be the case. What exactly may be happening in the selection of strains is that only those strains are being chosen in which some of the precursors that are used for penicillin synthesis are utilized with much less facility for the cell protein synthesis than in the wild strains. On the basis of this a picture can be formed of the cellular mechanism involved in the penicillin biosynthesis as follows:



L-cysteine being an amino acid is involved in the cell protein synthesis normally. Dimethylpyruvic acid or dihydroxyisovaleric acid are precursors of

the amino acids, valine or isoleucine which are also involved in protein synthesis; the two acids should be used up for penicillin synthesis before they are converted into the amino acids. When penicillin synthesis takes place, the cell protein synthesis is slowed down, these materials accumulate in the cell and get converted into penicillin by self condensation or by the mediation of some enzyme systems, which have not been identified so far by the author. Thus penicillin synthesis is not an all-or-none step, but one which is competitive with, and takes the upper hand over, the cell protein synthesis.

It is known how L-cysteine arises. The origin of dimethylpyruvic acid or dihydroxyvaleric acid has to be traced definitely and a probable course has been indicated. If acetate is a precursor for this unit, then the role of the carbon constituents that are added, may be only to provide this moiety in addition to providing the energy required to keep the metabolism going without the cell getting autolyzed. Ammonia is a metabolic product that can arise by the oxidative deamination of the amino acids in the medium. It stands to reason that the judicious addition of ammonium acetate in boosting up penicillin production has to be investigated. The role of the precursor acids for the synthesis of the side chains has been satisfactorily elucidated. It requires to be unravelled how these reactions are to be integrated and mediated for the effective synthesis of penicillin. It is also to be understood how the various conditions enumerated in the beginning as being associated with penicillin synthesis come into the picture.

One thing, which may sound paradoxical, is very clear. For effective penicillin synthesis what is required is the slowing down of reactions that are involved in the cell material synthesis itself. Since the mycelia are indispensable for the synthesis of penicillin, a mutant cannot be had in which there would be a block in the conversion of cysteine or the other acid into protein, because this would mean a stoppage of the mould growth itself. So, what is done is to get the maximum growth first, and then create a condition when this reaction does not go on at all, so that there is an accumulation of the precursors required for the penicillin synthesis. If this be the case, the question arises how does the poor penicillin producer differ from a good penicillin producer?

The other question of practical interest is: can the above-mentioned knowledge be made use of to enhance penicillin production by adding the specific precursors to the medium? Unfortunately the issue is complicated. It is known that the precursors are not inert metabolically but are metabolized to a very great extent and only 2-4 per cent of what is added is actually used up for penicillin synthesis. The same is also the case with phenylacetic acid. So, to make available more of the metabolites, L-cysteine or dimethylpyruvic acid, about 25 to 50 times the actual quantity required for penicillin synthesis has to be added to the medium. What would be the effect of such an excess of the compound on the normal rate of metabolism should be found out. There is a risk that a great excess of cysteine may even destroy the penicillin produced. Then the other questions which crop up are: how cheap would be these additions. Can the dimethylpyruvic acid be made available by better



biochemical processes? And if the use of the carbon sources is mostly to provide acetate, are there not better ways of providing them?

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### Discussion

DR. S. K. BOSE: Studies of the biochemical properties of the mutants of *P. chrysogenum* may give us a clue in determining the intermediates in the biosynthesis of penicillin. Has any work been done on this?

DR. K. GANAPATHI: Studies as mentioned have given very valuable results in other cases and have enabled us to trace the chemical steps involved in the biosynthesis of many compounds occurring in nature. But the case of penicillin appears to be different. No work has been done in this direction.

DR. P. N. NANDI: It was stated by the author that in the selection of strains involving mutagenic agents there may occur blocks in the various gene controlled steps and lead to the accumulation of various intermediate products in the reaction chain. Does penicillin form one such intermediate in a chain and is its increased production a result of a blocking of a synthetic step?

DR. K. GANAPATHI: In analogy with the work on neurospora mutants and other micro-organisms, we can visualize a reaction chain as follows with penicillin as an intermediate:



If the step penicillin to D is blocked by the loss of the gene controlling this step, then there would be an accumulation of penicillin in the medium when such a mutant is used. In fact, such appeared to be the thinking of Tatum, Beadle, etc. when they started the strain selection programme. But the facts we have, do not support this concept for the following reasons: (i) penicillin synthesis by the mould is not an all-or-none step as it has to be, if the above scheme is accepted ; better strains give more of penicillin than the poor ones ; (ii) penicillin appears to be an end product of a metabolic step gone wrong and in its synthesis the reaction chain has reached a blind alley ; from our present knowledge, it is difficult to visualize a metabolic reaction chain from penicillin ; and (iii) the precursors involved in penicillin synthesis are those connected with the growth of the mould and if there is a complete block of

this reaction, there would be no growth of the mould at all. In view of all these, the scheme suggested in the paper appears to be more probable.

DR. P. S. SARMA: Vitamins and inorganic elements may play a role in the biosynthesis of penicillin. So it may be worth while to study the actions of antivitamins and metal chelating agents which will inhibit some specific step in the synthetic chain.

DR. K. GANAPATHI: This would be an attractive method to follow but our difficulty is that we know very little about the steps and systems involved, so we cannot even select out specific agents. I would like to know whether there are specific agents that could inhibit the conversion of cysteine into cellular proteins or dimethylpyruvic acid into valine. I am not aware of any and it would be interesting to study such inhibitors in penicillin synthesis.

DR. V. SUBRAHMANYAN: Since we seem to recognize some compounds as precursors of penicillin, is it not possible to add large amounts of these to the fermenting medium and see if penicillin is synthesized in large quantities?

DR. K. GANAPATHI: This is exactly what occurs to anyone engaged in production. But unfortunately things are a bit complicated because of the fact that the mould metabolizes a very large percentage of what is added and only about 1-2 per cent is used for the synthesis of penicillin. For example, for the production of 10 kg. of penicillin, we would require on the stoichiometric basis about 3.45 kg. of dimethylpyruvic acid, 3.63 kg. of L-cysteine, 0.51 kg. ammonia and 4.08 kg. phenylacetic acid. But we may have to add to the medium, gradually or all at once, about 60-70 kg. dimethylpyruvic acid and about the same quantity of L-cysteine. Leaving aside the feasibility of adding such large amounts, the question of what would be the effect of these on the mould metabolism should be considered. It is known that cysteine inactivates penicillin and such a large amount is bound to destroy penicillin produced.

# Synthesis of Symmetrically Disubstituted Ethylene Diamine Dipenicillin G Salts

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Some N : N'dialkyl and nuclear substituted dibenzylethylenediamines and their dipenicillin G salts have been prepared and characterized. The bioassay of the penicillin salts have also been carried out.

The administration of penicillin in a satisfactory way still poses some problems because of the fact that penicillin is not very stable in the stomach and after injection it is excreted very fast<sup>1</sup> from the system. Attempts have been made to slow down this high rate of excretion in three ways.

One method is to administer sodium or potassium penicillin simultaneously with *p*-aminohippuric acid<sup>2</sup> or caronamide<sup>3</sup> (*p*-carboxy-phenyl-methane-sulphanilamide) and Benemid, *p*-(di-*n*-propyl sulphamyl)-benzoic acid<sup>4</sup>, which compete with penicillin in the renal tubular excretion and delay its excretion. Though very high blood levels of penicillin can be attained by this method, it is not practicable for mass trials.

The second method is to coat the penicillin with some compounds like protein material or soap detergent solution which allows penicillin to be absorbed slowly and prevent it from the destruction by the acid digestive juices in the stomach when given orally. For the parenteral administration, sodium or the calcium penicillin was given mixed with arachis oil and beeswax<sup>5</sup>. The thick oil suspension forms a depot from which penicillin slowly leaks out and gets distributed in the system. However, the injection of this type of viscous suspension gives considerable pain to the patient. Other derivatives possessing prolonged therapeutic effect recently described are zinc penicillin<sup>6</sup>, hexamine cobalt benzyl penicillinate trihydrate<sup>7</sup> and boron penicillin<sup>8</sup>. The use of sodium citrate or buffered potassium salt also prevents the rapid loss of potency<sup>9</sup>.

The third general method consists in the use of sparingly soluble salts of penicillin G with some organic bases which slowly liberate penicillin into the blood stream and maintain the effective blood levels for a long time.

In 1948, the sparingly soluble procaine salt of penicillin G<sup>10,11</sup> was introduced for use as one which maintains adequate blood concentration for a little longer time than penicillin. The injection of procaine penicillin in oil suspension is associated with pain and some local reactions. This oily suspension with 2 per cent aluminium monostearate is used for mass treatment of syphilis.



Later on, the use of procaine penicillin along with some dispersing agent in aqueous suspensions<sup>12</sup> was introduced as a definite improvement. The recent development is the introduction of a suspension of procaine penicillin containing some potassium penicillin to give an immediate high blood level which is then maintained by the slower action of procaine penicillin. The 2-chloro procaine salt<sup>13</sup> of penicillin is claimed to have advantages over procaine salt but this has not displaced procaine penicillin from practical use. L-Ephedrine salt<sup>14</sup> of penicillin G has been suggested as an alternative to the procaine salt but it seems to be less effective.

L-Ephenamine penicillin G<sup>15</sup> is one of the salts of penicillin which is claimed to reduce the potential allergic reactions due to penicillin. Similarly 1-1-dimethyl-2-hydroxy-propyl amino penicillin<sup>16</sup> is claimed to be a stable salt producing prolonged blood levels of penicillin whether given orally or by injection.

Vanderhaege<sup>17</sup> has described the sparingly soluble penicillin salts of methyl to *n*-amyl esters of phenylalanine. Out of the penicillin salts of dialkylamino alkylbenzoates prepared, only the diethylamino ethyl-*p*-hydroxybenzoate salt has the sparing solubility to the same degree as procaine penicillin.

The hydriodide of the diethylamino ethylester<sup>18</sup> of penicillin G shows marked affinity for pulmonary tissues and is tried for use in penicillin sensitive lung infections even though it is slightly more toxic than procaine salt. N-N' Dibenzyl ethylene diamine dipenicillin G<sup>19</sup> (Benzathine penicillin or Bicillin) is a new repository form of penicillin G now come into vogue. It is tasteless, quite stable in aqueous suspension and has toxicity comparable with that of procaine penicillin. It is soluble in water to the extent of 0.02 per cent and has potency of 1,200 u./mg. After intramuscular injection of 6,00,000 units therapeutic levels were maintained for as long as 28 days. It can be used satisfactorily for the infections which generally respond to penicillin except in acute infections or where higher blood levels are required immediately. This is well adapted for oral administration.

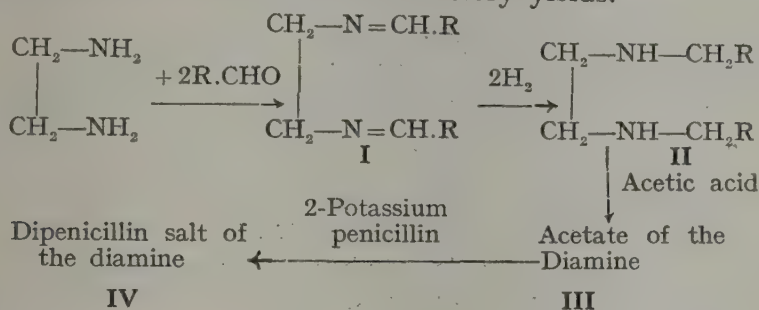
The other repository penicillin salts reported in recent years with claims of good results are: dibenzylamine penicillin G<sup>20</sup>, hydrabamine [N:N'-bis-(dehydroabietyl) ethylenediamine] penicillin G<sup>21</sup> and N-benzyl- $\beta$ -phenylethylamine penicillin G (Benethamine penicillin)<sup>22</sup>.

The present work is concerned with the preparation of stable salts of penicillin which after administration would maintain a penicillinemia of about 0.5 to 0.6 u./ml. for about a week. With such a salt even acute infections could be tackled satisfactorily. Benzathine maintains only 0.02 to 0.06 u./ml. for some weeks<sup>23,24</sup>. Taking this compound as a starting point an attempt is being made to find out the effects of introducing different types of substituents in the benzene ring of N:N'-dibenzyl ethylenediamine on the degree of absorption, distribution, etc., of these penicillin salts. A number of penicillin salts have thus been prepared and studied.

Penicillin salts of some of the bis-alkyl ethylene diamines are being prepared with alkyl chains varying from C<sub>1</sub> to C<sub>10</sub> and preliminary experiments show

that the solubility in water decreases as the alkyl chain increases. Further study is in progress.

There are three general methods of preparing N-N'-symmetrically disubstituted ethylene diamines: (i) condensation of ethylene diamine with aliphatic or aromatic halide; (ii) condensation of ethylene dihalide with aliphatic or aromatic amines; and (iii) preparation of Schiff's bases from ethylene diamine and the aldehyde which by catalytic reduction or sodium alcohol reduction give the corresponding secondary amines. All the methods were tried and it was found that by the first two methods a mixture of mono- di- and tetra-substituted derivatives was obtained from which it was difficult to separate the components. By the reduction of the Schiff's base with sodium and alcohol the pure products were obtained in satisfactory yields.



Because of the instability of penicillin G the problem of preparing the penicillin salts in the pure form was a bit difficult. There are two general methods for this. According to the first method, penicillin G is liberated from sodium or potassium salt by acidification of an aqueous solution and immediately extracted into the solvent. The dry solution of the base in the same solvent is mixed with the dry solution of penicillin when the penicillin salt separates out. The second method is to add the concentrated solution of the sodium or potassium salt of penicillin in water with or without some solvent like acetone or alcohol to an aqueous solution of the base hydrochloride or any acid. In the present studies, the latter method was found suitable. The aqueous solutions of the diacetates of amines were treated with solutions of the potassium salt of penicillin at 45° to 50°C. when the penicillin salts separated out in a crystalline form. These salts are quite stable, easily soluble in methyl alcohol and soluble with difficulty in acetone. The salts prepared are being tested for their absorption and excretion in rabbits.

#### EXPERIMENTAL AND RESULTS

##### N-N'-bis (substituted benzalidene) ethylene diamines (I)

Ethylene diamine (75%, 0.1 mol.) was mixed gradually with the aromatic aldehyde (0.2 mol.) at room temperature with shaking. The reaction was exothermic. It was heated on steam bath for 4-5 hr., cooled and crystallized from minimum quantity of alcohol. The yields ranged from 85 to 90 per cent.

##### N-N'-bis (substituted benzyl) ethylene diamines (II)

Sodium (0.8 mol.) was gradually added to a boiling solution of the above Schiff's base (0.1 mol.) in absolute ethyl alcohol (10 cc./g.) with constant stirring within half an hour. The stirring and heating on water-bath were



continued for another two hours. The reaction mixture was cooled and diluted with water after the alcohol was removed. The thick liquid that separated was extracted with ether and dried over potassium hydroxide. The solvent was recovered and the amine was distilled under reduced pressure (2-3 mm.), and was characterized by the preparation of the dihydrochloride in dry ether solution.

In the case of veratrylamine, the reduction was carried out in presence of amyl alcohol and the amine was isolated in the form of dihydrochloride by adding concentrated hydrochloric acid to the residue remaining after separating aqueous layer and removing amyl alcohol under reduced pressure. The dihydrochloride crystallized from ethyl alcohol in white leafy crystals, m.p. 261°C. (reported m.p. 250-55°C.)<sup>25</sup>.

### N-N'-bis (substituted benzyl) ethylene diamine diacetates (III)

The above amine (0.1 mol.) dissolved in the minimum quantity of dry ethyl alcohol and glacial acetic acid (0.21 mol.) was added with shaking at room temperature. The mixture was kept at room temperature for 2 hr. when the white crystals of diacetate separated. It was filtered and washed with dry acetone. The addition of acetone to the mother liquor gave further quantities of the diacetate. It does not require further purification and can be used as such for the preparation of the penicillin salts.

### N-N'-bis (substituted benzyl) ethylene diamine dipenicillin G (IV)

The filtered solution of the above diacetate (0.005 mol.) in distilled water (60 cc.) was added dropwise to the filtered solution of potassium penicillin G (0.01 mol.) in water (30 ml.) and acetone (30 ml.) at 45-50°C. with constant stirring. After the addition was over, the stirring was continued for 2 hr. when crystals of penicillin salt separated. The reaction product was cooled in ice bath, the crystals filtered, washed with water, and then with acetone and dried in air. Finally it was dried in vacuum at 45-50°C. Most of the products were quite stable. In the case of veratrylamine, dihydrochloride was used for the preparation of the salt.

The compounds obtained with their various characteristics are given in Table 1.

TABLE 1—CHARACTERISTICS OF SOME ETHYLENE DIAMINE DIPENICILLIN G DERIVATIVES

	AMINE (II) BASE, b.p., °C.*	DERIVATIVES OF AMINE (II), m.p., °C.			DIPENICILLIN G (IV)		
		Schiff's Base (I)	Diacetate (III)	Dihydro- chloride	Moisture %	m.p.† °C.	Potency u./mg.
Phenyl	182	52-53†	112†	298-300†	6.9	139†	1,165
<i>o</i> -Methoxy- phenyl	215-220	110-112†	96	168-170		109-11	1,155
<i>m</i> -Methoxy- phenyl	223-226	56	113	221-223	7.0	121	1,176
<i>p</i> -Methoxy- phenyl	220-230	106†	120	287†	7.5	127	1,163
3:4 Dimethoxy- phenyl		154-156†		261†		128	1,187

\* At 2-3 mm.

† Penicillin salts melt with decomposition

‡ As reported in literature



## ACKNOWLEDGMENT

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## Discussion

DR. J. N. TAYAL: Since the penicillin salts are reported to have 6.9-7.5 per cent moisture, will they not deteriorate due to the high moisture content?

DR. K. GANAPATHI: The deterioration of the salt depends upon the stability of the salt and not merely on its moisture content. For example, benzathine penicillin is stable in aqueous suspension for about two years.

DR. K. S. SANJIVI: For acute infections, we prefer to use the sodium or potassium salts. What is required is to have a local anaesthetic that will lessen the pain on injection. The repository salts are searched for to maintain prolonged blood concentrations of penicillin. The question is whether it is necessary at all to maintain this concentration for long. It may be that a higher concentration for shorter period may be enough to get rid of the invading pathogen.

# Attempts at Biosynthesis of Citrinin Analogues

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A probable mechanism of citrin biosynthesis was postulated and its validity tested by studying the influence of the hypothetical intermediates on citrinin elaboration by *Aspergillus candidus*. With the object of increasing the activity and lowering the toxicity, biosynthetic modifications of citrinin were also tried by introducing structurally related intermediates and derivatives of degradation products in the fermentation medium. The products were examined for melting point, antibacterial activity and bioautographic behaviour.

Biosynthesis of a metabolite involves a number of stepwise reactions, each step in the synthetic series being governed by a gene. Tatum<sup>1</sup> has postulated a scheme which accounts for the formation of C<sub>2</sub>, C<sub>3</sub> and C<sub>4</sub> fragments followed by secondary condensation of unicyclic rings, oxidations, chain syntheses and other processes, eventually resulting in quinones and other products.

The probability was considered that citrinin is derived from the condensation of 2:3-butylene glycol, malonic acid, pyruvic acid and *n*-propanol (as its aldehyde), products which are common in bacterial and mold metabolism<sup>6-8</sup> (Table 1). The hypothetic pathway of citrinin biosynthesis is given in Scheme 1.

## EXPERIMENTAL

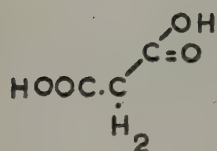
The modified Czapek-Dox medium of Raistrick and Smith<sup>3</sup> was fortified with 20 p.p.m. zinc sulphate (ZnSO<sub>4</sub>·7H<sub>2</sub>O) and sterilized in Roux bottles at 15 lb. pressure for 15 min. The postulated intermediates and other compounds were incorporated in the medium under appropriate conditions. After inoculation with spores of *A. candidus* and incubation at 37°C. for 21 days, the fermenting medium was filtered and the metabolic product was precipitated by adjusting the pH to 2 with hydrochloric acid. The precipitate was filtered, washed free from acid, dried *in vacuo* and weighed. It was then purified from dioxane according to the method of Tauber *et al.*<sup>4</sup>. Yield of citrinin varied with the period of incubation and, in 21 days, different batches gave values from 1.76 to 2.94 g./l. Results reported here represent average values of replicates from pooled lots.

The products obtained were tested for antibacterial activity by the serial dilution method using *Staphylococcus aureus* F.D.A. 209 as the test organism. The melting points of the products were determined and also the change, if any, in the melting points on mixing with pure citrinin.

TABLE 1—EFFECT OF 2:3-BUTYLENE GLYCOL, MALONATE AND *n*-PROPANOL ON CITRININ ELABORATION

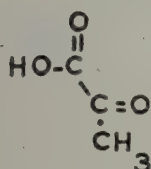
(Period of incubation, 21 days)

ADDITION	CONCENTRATION IN MEDIUM mg./l.	YIELD g./l.
None	..	2.70
Malonic acid	100	2.85
	200	2.50
	400	1.80
2:3-Butylene glycol	100	2.75
	200	3.00
	400	3.40
<i>n</i> -Propanol	200	2.70
	400	2.40



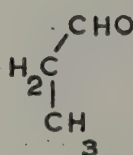
Malonic acid

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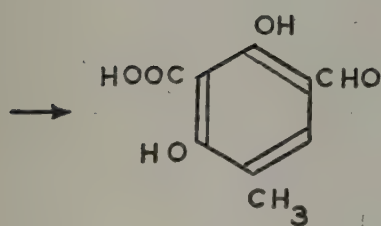


Pyruvic acid

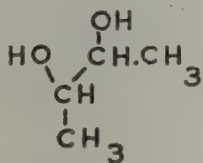
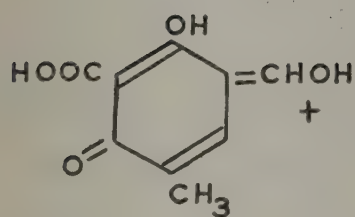
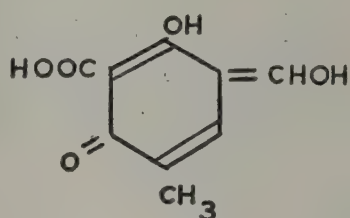
+



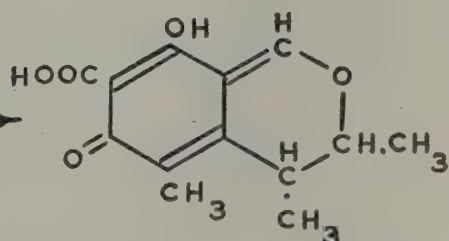
Propionic aldehyde



I

 $\rightleftharpoons$ 

2:3-Butylene glycol



Citrinin

SCHEME I



Bioautography of citrinin and of metabolic products was tried using butanol-acetic acid-water system and ascending technique of irrigation for separation on paper and peptone-glucose agar seeded with *Bacillus subtilis* for detection of the compounds. Although as little as 10  $\mu$ g. of citrinin can be detected, 100  $\mu$ g. of the products were spotted. Citrinin gave an Rf value of 0.68.

## RESULTS AND DISCUSSION

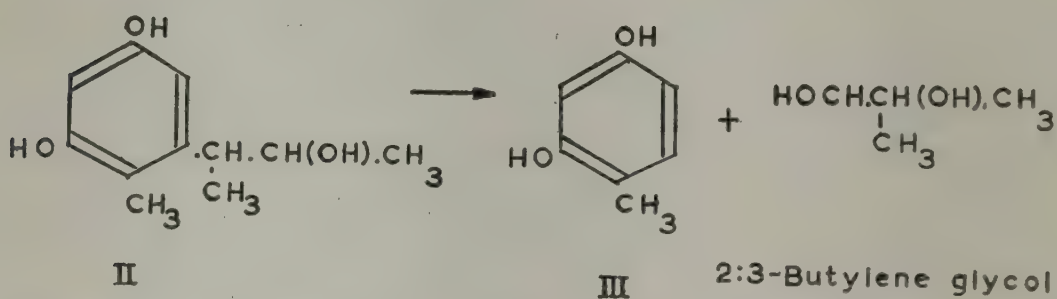
2:3-Butylene glycol stimulated citrinin production at all levels ; malonate showed a favourable effect at low concentrations, but depressed the yield at higher levels ; *n*-propanol had no effect on citrinin elaboration.

In addition to these compounds, acetate and citrate were considered as other possible intermediates. Besides, there is evidence to show that in mould metabolism citrate can give rise to malonate<sup>6</sup>, postulated above as an intermediate in citrinin biosynthesis. The results recorded in Table 2 indicate that with increase in acetate level there is progressive increase in citrinin yield ; citrate stimulation is also observable although, at higher concentration, citrate lowers the yield of citrinin.

### Use of derivatives of 4-*n*-hexylresorcinol

Derivatives of 4-*n*-hexylresorcinol have powerful antibacterial properties and are well tolerated by experimental animals. It was thought that the introduction of a hexylresorcinol nucleus in the citrinin molecule may improve its properties.

The phenolic alcohol (II) is obtained by the alkaline or acid hydrolysis of citrinin<sup>9,10</sup>. Condensation of 4-methyl-resorcinol (III) with 2:3-butylene glycol, if effected by a reagent such as boron trifluoride, is likely to result in substitution at the remaining  $\beta$ -position of the resorcinol nucleus, and not in the  $\alpha$ -position which is necessary for the formation of (II). It was improbable that the addition of 4-*n*-hexylresorcinol or the  $\gamma$ -carboxylic acid (IV) to the growth medium, even if the organism could be acclimatized to their presence, would lead to the production of a hexyl analogue of citrinin. Preliminary experiments confirmed this anticipation.



The replacement method was adopted since the compound suppressed the growth of the mould, and after a short period of incubation a much reduced yield of citrinin was obtained as seen from the results in Table 3.

When the amide of the acid (IV) was used in cultures of *A. candidus* at

TABLE 2—EFFECT OF ACETATE AND CITRATE ON CITRININ ELABORATION  
(Period of incubation, 26 days)

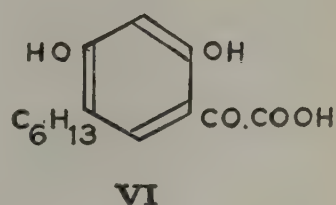
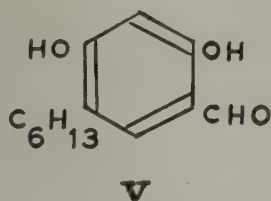
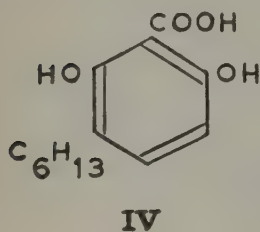
ADDITION g/l.	YIELD g/l.	INCREASE %
Nil	3.32	..
Acetate 0.5	3.44	3.7
Acetate 1.0	3.95	19.0
Citrate 0.5	3.92	18.2
Citrate 1.0	3.63	9.3

TABLE 3—EFFECT OF 4-n-HEXYLRESORCINOL-2-CARBOXYLIC ACID (IV) IN REPLACEMENT CULTURES

QUANTITY OF ACID mg. %	QUANTITY OF 2 : 3-BUTYLENE GLYCOL mg. %	PERIOD OF INCUBATION days	YIELD g./l.
40	15	13	0.62
60	23	10	0.48

pH 6.5 the yield of citrinin was 0.74 g./l. as compared with 2.9 g./l. in the control.

If the second  $\beta$ -position is blocked as in the aldehyde (V) and glyoxylic acid derivative (VI), condensation in the laboratory will be inhibited or take place in poor yield in the  $\gamma$ -position or in the  $\beta$ -position by displacement of the aldehyde or oxalyl group. The somewhat remote possibility of the intervention of (V) and (VI) in the biosynthesis of citrinin type was examined but the only product was citrinin in diminished yield.

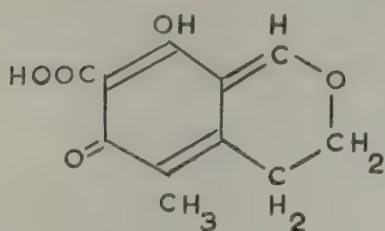


The mixed melting points with pure citrinin showed no change ; the anti-bacterial activities of the products against *S. aureus* were somewhat lower than 1:80,000, the value for pure citrinin. Bioautographic behaviour was similar to that of pure citrinin.

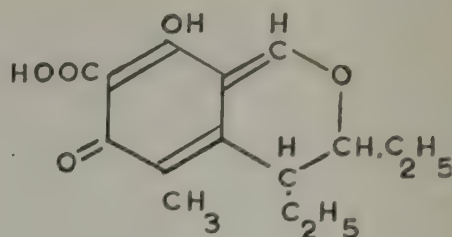
### Use of glycols

Ethylene and propylene glycols were used in the growth medium in the hope of producing the citrinins (VII) and (VIII). At levels of 1.6 and 1.4 g./l. respectively, ethylene and propylene glycols gave 3.0 and 2.55 g./l. of crude

citrinin melting at 165-167° (decomp.) as compared to 2.2 g./l. in the control set.



VII



VIII

Ethylene glycol, when used as the calcium salt of the phosphoric acid ester increased the yield of citrinin from 1.66 to 2.94 g./l. when the concentration

TABLE 4—EFFECT OF 4-n-HEXYLRESORCINOL-6-GLYOXYLIC ACID (VI) AND 4-n-HEXYLRESORCINOL-6-ALDEHYDE (V)

(Period of incubation, 31 days)

ADDITION	CONCENTRATION IN MEDIUM mg. %	YIELD g./l.	MELTING POINT (decomp.) °C.
None	..	3.8	170
Acid (VI)	40	3.3	164
Aldehyde (V)	15 30	3.45 3.10 }	163-164

TABLE 5—EFFECT OF AMINO ACIDS

(Period of incubation, 21 days)

AMINO ACID	CONCENTRATION g./l.	YIELD g./l.	MELTING POINT (decomp.) °C.
None	..	2.22	170
<i>l</i> -Leucine	0.5	2.67	165-67
<i>dl</i> -β-Phenylalanine	0.5	2.64	167
<i>l</i> -Tyrosine	0.5	3.03	169

TABLE 6—EFFECT OF PHENYLACETIC ACID

(Period of incubation, 21 days)

ADDITION	ORGANISM	INCUBATION TEMPERA- TURE °C.	YIELD g./l.	MELTING POINT (decomp.) °C.
None	<i>A. candidus</i>	37	2.90	170
Phenylacetic acid	<i>A. candidus</i>	37	2.68	165
None	<i>P. sartoryi</i> NRRL 783	30	0.48	168
Phenylacetic acid	<i>P. sartoryi</i> NRRL 783	30	0.60	166



of the calcium salt was increased to 1 per cent ; it also gave appreciably good yields of the product (3.07 and 1.92 g./l. respectively) when added as the sole source of phosphate at 0.6 g./l. to cultures of *A. candidus* and *Penicillium sartoryi*<sup>11</sup>. In all the above cases, however, the antibacterial activity and bioautographic behaviour of the products were the same as those of pure citrinin.

### Use of amino acids

In the biosynthetic scheme outlined earlier, pyruvic acid is a participant. Since it was found to be toxic to the organism and since formation of  $\alpha$ -keto acids by oxidative deamination is known in mould metabolism<sup>12,13</sup>, a biosynthetic modification of citrinin was attempted by the use of some amino acids in the medium. The results are shown in Table 5.

The amino acids tried stimulated citrinin elaboration as a result of increased growth, the maximum effect being produced by tyrosine.

### Use of phenylacetic acid and fatty acids

Stimulation of citrinin elaboration by acetate suggested that acetate may contribute to one or more methol groups in the citrinin molecule. Phenylacetic acid was therefore used as a precursor of modified citrinins. *n*-Caproic acid, *n*-capramide, adipic acid and dodecylamine were used in order to introduce higher fatty acid chains in the molecule.

TABLE 7—EFFECT OF FATTY ACIDS

(Period of incubation, 21 days)

ADDITION	CONCENTRATION IN MEDIUM g./l.	YIELD g./l.	MELTING POINT (decomp.) °C.
None	..	2.26	170
Adipic acid	0.5	2.17	168
Ammonium- <i>n</i> -caproate	1.86	0.39	166
	1.10	0.46	166
<i>n</i> -Capramide	1.25	0.34	168

TABLE 8—EFFECT OF DODECYLAMINE HYDROCHLORIDE

(Period of incubation, 21 days)

ADDITION	ORGANISM	INCUBATION TEMPERA- TURE °C.	YIELD g./l.	MELTING POINT (decomp.) °C.
None	<i>A. candidus</i>	37	2.94	170
Dodecylamine hydrochloride	<i>A. candidus</i>	37	1.06	165
None	<i>P. sartoryi</i> NRRL 783	30	0.60	167
Dodecylamine hydrochloride	<i>P. sartoryi</i> NRRL 783	30	0.12	165

Since phenylacetic acid was toxic to the mould at pH 4.5<sup>14</sup>, it was added to 4 days old culture of *A. candidus* and to 7 days old culture of *P. sartoryi* grown in media at pH 6.5. The results given in Table 6 reveal that the compound does not affect the yield of the product. Phenylacetic acid, when used in replacement cultures of *A. candidus* at a level of 1 g./l., gave 1.96 g. of the product as compared to 2.28 g. of citrinin in the control.

Results with fatty acids (Table 7) reveal that, unlike *n*-caproic acid and its amide, adipic acid has no effect on the yield.

Dodecylamine was very toxic to the mould and was used as the hydrochloride at 20 mg. per cent. With 6-days-old cultures of *A. candidus* the results in Table 8 show marked decrease in the yield.

Although the metabolic products obtained with capric acid, capramide and dodecylamine hydrochloride showed a reduced activity, there was no change in the mixed melting points with pure citrinin and the bioautographic behaviour was also the same as that of pure citrinin.

From the foregoing experiments it appears that of the postulated intermediates in citrinin biosynthesis, only 2:3-butylene glycol and malonate in small concentrations stimulated citrinin elaboration, indicating a probable precursorial role for these compounds. The suppression of citrinin elaboration by malonate at higher levels may probably be due to inhibition of succinic oxidase activity of the mould, as was partly evident by a marked shrivelling of the mycelium. It is quite probable that citrate exerts its effect by providing malonate: its decreased stimulation at higher level may be due to accumulation of malonate, sufficient to slow the respiration of the mould.

The inability of the organism to form modified citrinins using glycols indicates that they are probably split into C<sub>2</sub> and C<sub>3</sub> products before they are utilized for citrinin synthesis. The fairly good yields of citrinin with the phosphoric acid ester of ethylene glycol as the sole source of phosphate may suggest that the organism utilizes this compound through phosphatase action, the ethylene glycol released stimulating citrinin elaboration.

Most of the intermediates used in the attempted biosynthesis of citrinin analogues, especially 4-*n*-hexyl-resorcinol derivatives, caproic acid, capramide and dodecylamine markedly suppress growth and hence citrinin elaboration. From the results it seems that the compounds are not utilized intact by the organism, but are metabolized in other ways.

There was a stimulation of growth followed by increased elaboration of citrinin with the use of amino acids, indicating that these might be used by the organism for energy requirements and formation of cellular proteins, but not in the synthetic steps in citrinin formation.

It is recognized that the present studies are extremely inadequate for determining the nature of the precursors in citrinin elaboration by the organism. More useful indications can doubtless be obtained by the use of (i) labelled metabolites followed by identification of the positions in the antibiotic in which the label has been incorporated and (ii) derivatives or analogues of postulated intermediates labelled in specified positions whose path is traceable in citrinin molecule.

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# Fermentation Products of *Penicillium herquei* : A Preliminary Study

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Chemical and microbiological observations on fermentation of *P. herquei* Bainier & Sartory in surface and deep cultures in shake flasks have been made. Antibiotic properties and the nature of the pigments have been studied. Interesting effects of the various trace elements on the morphology of the fungus and nature of the pigments produced by it are recorded. Possible interpretations are discussed. The chemical nature of the fermentation products with special reference to the pigments is under study.

Raper and Thom<sup>1</sup> divide the genus *Penicillium* into four big groups on the basis of the nature of the penicillus. According to them, *P. herquei* series, although widely distributed, is not abundant and represents one of the easily placed and the most colourful of the genus. A few physiological and biochemical reports have been made by Stodola *et al.*<sup>2</sup>, Harman *et al.*<sup>3</sup>, and Galarraga *et al.*<sup>4</sup> Burton<sup>5</sup> has reported herquein, an antibiotic principle active against *Shigella* from the culture filtrates of *P. herquei*. The product obtained from the fermentation of *P. herquei*, isolated in soil samples from Ajmer appears to be different from what is reported by Galarraga *et al.*<sup>4</sup> and Harman *et al.*<sup>3</sup>

## EXPERIMENTAL

The organism under study could be readily placed in the *P. herquei* series on the basis of the biverticillate nature of the penicillus, coarse roughened conidiophores, vegetative mycelium typically in yellow shades with a dark reverse.

The growth of the fungus varies with the different identifying solid media, viz., Czapek-Dox, malt, and steep agars. While in the first it is restricted and attains a diameter of 2 to 3 cm. in two weeks, in the others it attains twice the diameter in the same time although the growth is more spreading. The colour of the reverse of the colony is yellow in Czapek's tending to marginal precipitation of a red pigment while in malt and steep agars it is predominantly green. Microscopic examination reveals that most of the colour is incrustated in the mycelium only although there is slight diffusion into the medium.

The fungus grows well on synthetic and natural liquid media like Czapek-Dox, corn-steep and groundnut meal media and also on mycelial hydro-

lysate of the filter wastage of penicillin fermentations. The pigment in synthetic medium is yellowish to red while in natural media it tends to be green. It grows well in submerged cultures and surface cultures, the type of pigment production being the same except that it is quicker in the submerged cultures. Thus while it takes over four weeks for the flasks to be ready for harvesting in surface cultures, the submerged cultures are ready in the course of five days. The time of harvesting is determined by the exhaustion of the sugars in the medium. At this stage pH is about 3.5.

All fermentations were carried out in liquid Czapek-Dox medium at 24°C. and submerged cultures were shaken in a rotary shaker giving 280 r.p.m. The inoculum was standardized to a 7-day old culture on a sporulating medium containing yeast extract. Spores were suspended in sterile water and distributed in the test flasks so as to get a concentration of  $3 \times 10^3$  per cc.

After growth, the mycelial mat in surface cultures, and the mycelium in submerged cultures were separated, dried and the pigment scraped out of the mats and mycelium treated with acetone to extract all the colour. While Galarraga *et al.*<sup>4</sup> have used ether, acetone has been found more suitable by the authors. It was possible to obtain crystals directly from the acetone solution. Solutions of the crystals in the various solvents and the culture filtrate, all give a brilliant green fluorescence in ultra-violet light.

TABLE 1—EFFECT OF TRACE ELEMENTS ON SUBMERGED CULTURES OF *P. HERQUEI*

TREATMENT	pH	MYCELIAL COLOUR	FLUORESCENCE	DRY WEIGHT	ANTIBIOTIC ACTIVITY
No trace elements	6.0	Brown	+	0.2123	Nil
Glucose only	6.0	White	—	Negligible	Nil
Glucose & NaNO <sub>3</sub>	5.0	Light yellow	—	0.1536	Nil
Czapek-Dox (Fe and Mg)	3.0	Deep yellow	+	1.4349	+
Cz + Fe, Mg, Zn	3.0	Incrusted yellow	+	1.3774	+
Cz + Mg, Zn	3.5	Deep yellow red ppt.	+	1.1500	+
Cz + Zn	5.5	Bright green	+	0.3395	+
Cz + Fe	5.0	Dark green	+	0.2283	+
Cz + Mg	5.5	Bright yellow	+	1.1265	+

Cz: Czapek's medium without trace elements

It was noticed that variation in trace elements like zinc, iron and magnesium induced striking variations in colour production, morphology and growth of the fungus. All shades of green, yellow and orange were seen in the presence of single or combined trace elements.

### RESULTS AND DISCUSSION

A summary of the results of a series of experiments on the influence of trace elements is presented in Table 1.

It is interesting that without trace elements the colour produced is negligible and no fluorescence is noted. It is evident from Table 1 that trace elements are necessary for growth and the growth is the highest in the presence of magnesium among the single elements. Also inorganic nitrogen and phosphate seem to be essential for growth. It is also interesting that the antibiotic activity is absent in the culture filtrates not receiving any trace elements. Further work in this direction is in progress. There appears to be slight inhibitory action by zinc.

All the antibiotic activity is found in the culture filtrates. It appears in the early stages of fermentation but is not found when the flasks are harvested. It is reasonable to assume that the pH which becomes quite low destroys the antibiotic. These findings are similar to Burton's<sup>5</sup> who reports herquein from the culture filtrates of *P. herquei*. Activity against *Staphylococcus aureus* and *Shigella shigae* are indicated in Table 1. Its activity is also noted against a number of soil protozoa and nematodes.

TABLE 2—PROPERTIES OF HERQUEINONE AND THE NEW COMPOUND

PROPERTIES	HERQUEINONE	NEW COMPOUND
Description	Brick red needles	Yellow and brownish rectangular rods
Melting point	224-226°C.	Decomposes above 250°C.
Solubility	Sol. in chloroform, ether, EtOH, ethyl acetate and insol. in light petroleum	Sol. in chloroform, ether, EtOH, Et acetate and insol. in light petroleum
Sodium hydroxide (2N)	Deep orange fading to pale yellow	Deep red soln. fading to pale yellow, with green fluorescence in ultra-violet light
Sodium carbonate solution	Insoluble	Soluble to form a yellow solution changing to green
Sulphuric acid	Orange solution with yellow fluorescence; on standing, green fluorescence	Yellow solution with green fluorescence
Ferric chloride solution	Intense black colour	Olive brown colour
Methylation in acetone with dimethyl sulphate	A fully methylated compound with no colour on addition of ferric chloride solution	Gives a methyl ether with a red colour on addition of ferric chloride solution



Circular paper chromatograms of the pigment spotted from acetone solutions and developed in butanol-acetic acid-water and isopropyl alcohol-water mixtures indicated two well defined components, a slow moving greenish blue pigment and a second fast moving yellow pigment. Transformation of yellow pigment to green both on exposure to light and on chemical treatment such as feeding certain trace elements in the nutrient medium has been observed.

A summary of the properties of the new substance and those reported by American and English workers is presented in Table 2.

It is evident from Table 2 that the new compound isolated by us is different from that reported in literature.

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# Preliminary Observations on the Mode of Action of Morellin

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The influence of some metallic ions, amino acids, vitamins, sera and serum fractions on the activity of morellin on *Micrococcus pyogenes* var. *aureus* has been studied.

The influence of metallic ions, amino acids and vitamins on the antimicrobial activity of morellin<sup>1</sup> has been investigated and preliminary observations are given in this paper.

Rao and Verma<sup>2</sup> previously observed that morellin gets inactivated in the system, probably as a result of binding with a fraction of the serum proteins which appeared to be  $\beta$ -globulin from electrophoretic mobilities. In order to get further clarification, the effect of addition of various sera and purified serum fractions on the activity of morellin have also been studied.

## EXPERIMENTAL

The organism used in these investigations is the Oxford strain of *Micrococcus pyogenes* var. *aureus* (obtained through the courtesy of Schimmel Culture Collection, Holland). The minimum inhibitory concentration (MIC) of morellin on this strain is 0.5  $\mu\text{g.}/\text{ml.}$

The serial dilution assays were carried out as described previously<sup>3</sup> in a deficient medium of Gale and Taylor<sup>4</sup>. The standard solutions of all substances except the sera and protein fractions were sterilized at 5 lb. pressure

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TABLE 1—EFFECT OF PHOSPHATES AND METALS ON THE ACTIVITY OF MORELLIN

	CONC./ml.	MIC ( $\mu\text{g.}/\text{ml.}$ )
Nutrient broth without phosphate	..	0.33
Nutrient broth with phosphate (M/30)	..	0.50
Medium + Cobalt chloride	10 $\mu\text{g.}$ 20 $\mu\text{g.}$	0.50 0.50
Medium + Ferrous sulphate	10 $\mu\text{g.}$ 20 $\mu\text{g.}$	0.50 0.75
Medium + Ferric chloride	10 $\mu\text{g.}$ 20 $\mu\text{g.}$	0.50 0.75

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for 15 min. before addition. The latter were incorporated into the medium after Seitz filtration.

The results obtained are recorded in Tables 1-4.

TABLE 2—EFFECT OF AMINO ACIDS ON THE ACTIVITY OF MORELLIN

	CONC. /ml.	MIC ( $\mu$ g. /ml.)
Medium + glycine	0.1 mg.	0.50
	0.2 mg.	0.50
Medium + serine	0.1 mg.	0.50
	0.2 mg.	1.06
Medium + threonine	0.1 mg.	0.50
	0.2 mg.	0.50
Medium + cysteine	0.1 mg.	0.33
	0.2 mg.	0.50
Medium + glutamic acid	0.1 mg.	0.50
	0.2 mg.	0.50
Medium + tyrosine	0.1 mg.	0.50
	0.2 mg.	0.50
Medium + lysine	0.1 mg.	0.50
	0.2 mg.	0.50
Medium + asparagine	0.1 mg.	0.50
	0.2 mg.	0.50
Medium + methionine	0.1 mg.	2.00
	0.2 mg.	3.30

TABLE 3—EFFECT OF VITAMINS AND OTHER SUBSTANCES ON THE ACTIVITY OF MORELLIN

	CONC. /ml.	MIC ( $\mu$ g. /ml.)
Medium + thiamine	0.1 mg.	0.33
	0.2 mg.	0.50
Medium + riboflavin	0.1 mg.	0.50
	0.2 mg.	0.50
Medium + nicotinic acid	0.1 mg.	0.50
	0.2 mg.	0.50
Medium + nicotinamide	0.1 mg.	0.50
	0.2 mg.	0.50
Medium + pyridoxine hydrochloride	0.1 mg.	0.33
	0.2 mg.	0.50
Medium + calcium pantothenate	0.1 mg.	0.50
	0.2 mg.	0.50
Medium + PABA	0.1 mg.	0.50
	0.2 mg.	0.50
Medium + folic acid	0.05 mg.	0.50
	0.10 mg.	0.50
Medium + B <sub>12</sub>	1.00 $\mu$ g.	0.33
	2.00 $\mu$ g.	0.33
Medium + biotin	0.4 $\mu$ g.	0.50
Medium + nucleic acids (yeast)	0.1 mg.	0.50
	0.2 mg.	0.50



TABLE 4—EFFECT OF SERA AND THEIR FRACTIONS ON THE ACTIVITY OF MORELLIN\*

	CONC. /ml.	MIC $\mu$ g./ml.
Medium + sheep serum	2.5%	1.00
	5.0%	2.00
Medium + rabbit serum	2.5%	1.00
	5.0%	2.00
Medium + rat serum	2.5%	1.00
	5.0%	2.00
Medium + serum albumin fraction 5	0.1 mg.	0.5
	0.2 mg.	1.0
Medium + $\beta$ -globulin Cohn Fr. 4	0.1 mg.	0.5
	0.2 mg.	1.0
Medium + $\gamma$ -globulin	0.1 mg.	0.5
	0.2 mg.	1.0
Medium + fibrinogen	0.1 mg.	0.5
	0.2 mg.	0.5
Medium + all the four fractions together	0.1 mg. each	1.0

\* Precipitation was observed in the first three tubes

### CONCLUSION

Cysteine, thiamine, pyridoxine hydrochloride and vitamin B<sub>12</sub> appeared to enhance the activity of morellin whereas only methionine considerably reduced the activity at the concentrations tried.

The activity of morellin was reduced to 2  $\mu$ g./ml. in the presence of the three sera accompanied by some precipitation. However, fibrinogen, serum albumin fraction 5,  $\beta$ -globulin Cohn Fr. 4, and  $\gamma$ -globulin in concentrations of 0.1 and 0.2 mg./ml. individually or in combination did not show any precipitation nor significantly reduced the activity.

Phosphate reduced morellin activity from 0.33  $\mu$ g./ml. to 0.5  $\mu$ g./ml. Co<sup>++</sup>; Fe<sup>++</sup> and Fe<sup>+++</sup> exerted no significant effect on the activity of morellin.

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# Antibiotics in Animal Nutrition : Mechanism of Growth-promoting Action of Aureomycin

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**Some studies on the growth-accelerating effect of aureomycin in rats kept on duck egg white (DEW) proteins (the utilization of which is inefficient owing to the presence of trypsin-inhibitor) have been made. Attempts have been made to throw some light on the mode of action of antibiotics (aureomycin) in accelerating animal growth in the light of the results obtained in this investigation.**

It has been well established that feeding of antibiotics has an accelerating effect on growth of various animals<sup>1,2</sup>. This property of antibiotics finds application in animal husbandry practice for enhancing the growth rate of animals and increasing feed utilization. The growth-accelerating effect varies from antibiotic to antibiotic, from species to species, and also depends upon the environment in which the animals are reared and the nature of the basal ration to which the antibiotic is supplemented<sup>1,2</sup>. Antibiotics have been shown to exert a highly beneficial effect on the growth of animals receiving all-plant rations<sup>7-8</sup>. These observations have been confined mainly to soya-bean, cotton-seed, groundnut meal and in one case rice. However, the beneficial effect of antibiotics on animal proteins like fish meal<sup>3,5,9</sup>, casein fed at low level<sup>10</sup> and also on a meal rich in animal protein<sup>11</sup>, has also been observed, though the effect is less than on all-vegetable rations. These interesting observations have raised the question of the mode of action of antibiotics. This knowledge is essential not only for the further application of antibiotics in animal nutrition, but for the appraisal of the nature of increased growth in animals fed antibiotics.

It has been suggested that antibiotics accelerate growth by preventing infection in animals<sup>12,13</sup> and by sparing the nutrients like vitamins<sup>14-16</sup> and amino acids<sup>5,8,17-20</sup> either by intestinal synthesis or by preventing their destruction by the intestinal micro-organisms. In view of the highly beneficial effect of antibiotics on all-vegetable rations which are deficient in some essential amino acids the concept of the protein sparing effect of antibiotics assumes significance. In all these cases a satisfactory and consistent hypothesis for the mode of action of antibiotics in accelerating animal growth is still lacking in spite of a mass of accumulated data.

## EXPERIMENTAL

Growth experiments were conducted with albino rats about four weeks old weighing between 40 and 50 g., distributed into groups of six each and housed in individual cages. The various litters were distributed evenly among different groups with respect to sex and initial weight. The rats were given different diets as described in Table 1.

In each experiment, for each diet, there was one control group not receiving aureomycin supplementation and a second which received aureomycin supplementation (100 mg./kg. diet). Growth was observed for a period of four weeks. A record of food intake was also kept.

In one of the experiments, rats kept on unautoclaved DEW with and without aureomycin, were placed in metabolic cages after 28 days, and faecal N excretion was determined on the corresponding diet over a four day period.

## RESULTS AND DISCUSSION

The results of feeding aureomycin on the growth of rats subsisting on different diets are given in Table 2.

It is seen from experiments 1 and 2 that aureomycin brought about a significant improvement in growth of rats on unautoclaved DEW while it had no

TABLE 1—DIETS FOR GROWTH EXPERIMENTS ON RATS

	DIET%					
	I <sup>1</sup>	II <sup>1</sup>	III	IV <sup>2</sup>	V	VI
Unautoclaved DEW	10	..	..	..	..	..
Autoclaved DEW	..	10	..	..	..	..
Casein	..	..	..	..	10	10
Casein hydrolysate <sup>3</sup> (enzymatic)	..	..	10	10	..	..
DEW ovomucoid <sup>3</sup>	..	..	..	1.0	..	1.0
Starch	81	81	81	80	81	80
Salt mixture <sup>4</sup>	4	4	4	4	4	4
Vitamin mixture <sup>5</sup>	0.1	0.1	0.1	0.1	0.1	0.1
Oil	5	5	5	5	5	5
Choline	0.1	0.1	0.1	0.1	0.1	0.1

<sup>1</sup> Diets were prepared as described by Narasinga Rao and Patwardhan<sup>21</sup>.

<sup>2</sup> Aminoxyl, Raptakos: Ca, 65% hydrolysed.

<sup>3</sup> Prepared from fresh DEW as described by Narasinga Rao and Patwardhan<sup>22</sup>.

<sup>4</sup> Salt mixture described by McCollum and Davis<sup>23</sup>.

<sup>5</sup> Thiamine 200 mg.; riboflavin 400 mg.; pyridoxine 200 mg.; nicotinic acid 400 mg.; calcium pantothenate 800 mg.; p-amino benzoic acid, 2,000 mg.; inositol 200 mg.; vitamin K 400 mg.; folic acid 40 mg.; vitamin B<sub>12</sub> 0.6 mg. These were made up to 20 g. The rats were given orally  $\beta$ -carotene, 100 I.U./rat/day; biotin 4  $\mu$ g./rat/day, vitamin D 15 I.U./rat/day and  $\alpha$ -tocopherol 4 mg./rat/day.

<sup>6</sup> To this diet aureomycin was supplemented after four weeks of growth when this group was divided into two groups of 3 rats each. One group received diet V and the other diet V plus aureomycin. Growth was observed for a further period of four weeks. Aureomycin did not have any effect.



TABLE 2—EFFECT OF AUREOMYCIN ON GROWTH OF RATS

(6 rats in each group; protein, 10% level)

EXPERI- MENT NO.	DIET	WEIGHT, g.			PROTEIN EFFI- CIENCY RATIO	't' VALUES
		INITIAL	FINAL	GAIN IN 4 WEEKS ± S.E.		
1	Diet I—Control	57.3	91.3	34.0 ± 3.62	1.23	1-2
	Diet I + 100 mg./kg. aureomycin	56.9	104.3	47.4 ± 3.70	1.76	3.04
	Diet II—Control	57.0	115.5	58.5 ± 2.52	2.22	
	Diet II + 100 mg./kg. aureomycin	56.1	108.7	52.6 ± 3.04	1.99	
2	Diet I—Control	53.5	73.5	20.0 ± 1.55	0.82	1-2
	Diet I + 100 mg./kg. aureomycin	53.8	87.7	33.9 ± 1.49	1.31	6.42
	Diet II—Control	53.7	112.0	58.3 ± 4.01	2.24	
	Diet II + 100 mg./kg. aureomycin	53.8	108.8	55.0 ± 1.79	2.08	
3	Diet III—Control	40.2	104.8	64.6 ± 2.04	2.52	1-2
	Diet III + 100 mg./kg. aureomycin	39.8	107.5	67.7 ± 1.75	2.65	1.54
	Diet IV—Control	39.3	80.5	41.2 ± 1.49	1.66	3-4
	Diet IV + 100 mg./kg. aureomycin	39.2	85.2	46.0 ± 1.71	1.80	2.11
4	Diet V—Control	45.2	117.5	72.3 ± 3.09	2.74	2-3
	Diet VI—Control	45.5	98.3	52.8 ± 2.03	2.00	1.87
	Diet VI + 100 mg./kg. aureomycin	45.0	103.0	58.0 ± 1.73	2.20	

such effect on the growth of rats subsisting on autoclaved DEW. As a matter of fact, aureomycin slightly decreased (not significant) the growth of rats on autoclaved DEW. These findings about aureomycin are similar to the findings of Hensley<sup>20</sup> *et al.* with raw and autoclaved soya-bean. However, the improvement in growth obtained by adding aureomycin to unautoclaved DEW is very much less than that which could be obtained by merely autoclaving the latter. Thus aureomycin only partially reversed the growth-depressing effect of unautoclaved DEW which has been traced to the presence of a growth inhibitor<sup>21</sup>.

Aureomycin, however, did not counteract the growth depression caused by DEW ovomucoid when fed to rats along with casein hydrolysate (Experiment 3, Table 2). There was only a slight improvement in growth when aureomycin was added to both diets III and IV, the extent of improvement being similar on both these diets. Similarly the effect of aureomycin on growth of rats receiving casein with DEW ovomucoid is also not significant (Table 2, Experiment 4).

Table 3 shows that aureomycin did not significantly alter the overall absorption of nitrogen from unautoclaved DEW. Carroll<sup>19</sup> *et al.* have made a similar observation with soyabean. Pecora<sup>6</sup> has observed that aureomycin, though it increased the protein efficiency of rice diets supplemented with threonine and lysine, did not affect the nitrogen retention on these diets. Berry and Schuck<sup>10</sup>, however, reported that although aureomycin improved the apparent digestibility of cotton-seed and soya-bean proteins, it impaired their utilization (nitrogen retention) following absorption.

The results indicate that the effect of aureomycin in improving growth is not confined only to vegetable proteins. The effect of the antibiotic must be only on protein utilization since the effect of ovomucoid in causing growth retardation is through interference with protein utilization alone and not due to interference with the availability of vitamins or minerals (authors' unpublished data). Hence it may be said that aureomycin may improve protein utilization which has been impaired by the presence of factors like trypsin inhibitor as in DEW and soya-bean.

Before discussing the mechanism of growth-promoting action of aureomycin as observed in these experiments, the mechanism of growth-depressing action of DEW ovomucoid will be briefly considered since both of them are inter-related. DEW ovomucoid would probably depress growth by two mechanisms:

1. This is due to the action of ovomucoid on the intact protein. Since DEW ovomucoid is a powerful antitrypsin it could interfere with the digestion of protein and liberation of amino acids from it. However, DEW ovomucoid, in spite of its powerful *in vitro* antitryptic activity, depresses the *in vivo* digestibility of only a few proteins (*viz.*, DEW proteins) whereas the effect on

TABLE 3—NITROGEN METABOLISM IN YOUNG RATS FED ON UNAUTOCLAVED DEW WITH AND WITHOUT AUREOMYCIN

(6 rats in each group ; protein, 10% level)

DIET	AVERAGE INTAKE g.	AVERAGE FAECAL N g.	AVERAGE APPARENT N ABSORBED g.	MEAN APPARENT DIGESTI- BILITY (%) $\pm$ S.E.	't' VALUES
Diet I—Control	0.8043	0.4572	0.3471	43.1 $\pm$ 7.67	1.9
Diet I + 100 mg./kg. aureomycin	0.8552	0.4217	0.4335	50.7 $\pm$ 8.23	

other proteins is negligible (*viz.*, casein). In those cases where the anti-trypsin is active *in vivo* the interference with protein utilization may take place in two ways: due to a decreased overall absorption of protein and due to slow rate of digestion. Under the latter circumstances a considerable portion may remain unabsorbed when it enters the lower end of the intestine and at this site the products of digestion are likely to be attacked by the intestinal bacteria, producing toxic products<sup>24</sup>.

2. This is the growth-depressing action of DEW ovomucoid when it is fed with a protein hydrolysate. The mode of this action is not yet clear. It is not due to antiereptic activity of DEW ovomucoid for it was found in *in vitro* experiments that ovomucoid did not inhibit the activity of erepsin. It is also not due to the toxic effect of ovomucoid since parenteral administration of ovomucoid did not have any effect on growth of rats. Since the ovomucoid action lies entirely in the intestinal tract and the growth-depressing effect of ovomucoid could be abolished by increasing the protein level in the diet, it is likely that ovomucoid interferes with the availability of amino acids from the protein hydrolysate, probably by altering the rates of absorption of amino acids.

The mode of action of aureomycin in promoting growth can be interpreted in the light of the above discussion. Since aureomycin is effective only when fed with an intact protein and that too a protein whose *in vivo* digestibility is affected by the ovomucoid and since it does not alter the overall nitrogen absorption, the following possibilities of aureomycin action can be considered: (i) Aureomycin may increase the rate of absorption of amino acids in intestine without altering the overall absorption; this requires the action of antibiotic on the enzyme systems involved in absorption; (ii) the presence of aureomycin may influence the intestinal flora so as to favour synthesis of some amino acids which would make good the loss due to lowered digestion; (iii) the presence of aureomycin may destroy deleterious bacteria from the lower intestine preventing the formation of toxic products and promoting the absorption of the products of digestion at this site. The first two possibilities are unlikely since growth acceleration has not been observed on diets IV and VI. The last mentioned possibility is the most likely one and is consistent with the antibacterial property of the antibiotic.

Finally, the question whether aureomycin induces true growth or only aids fat deposition has to be considered. Evidence presented by several workers<sup>25-29</sup> indicates that antibiotics induce weight gain in rats, dogs and pigs by enhancing fat deposition, rather than increasing the protein content of the body. Contrary evidence also has been published by Forbes<sup>30</sup>. However, in considering these data the fact that there are bound to be differences in the body composition of stunted and fast growing animals should not be overlooked<sup>31</sup>.

#### SUMMARY

Aureomycin significantly improves the growth of young rats kept on un-autoclaved duck egg white.



It does not significantly alter the growth rate of rats receiving the diets (i) casein with DEW ovomucoid or (ii) casein hydrolysate (enzymatic) plus DEW ovomucoid.

Nitrogen absorption from an unautoclaved DEW diet in young rats is not altered by the presence of aureomycin.

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# Interference with Amino Acid Metabolism by Citrinin

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**A study of metabolites causing reversal of citrinin action has been made. The amino acids arginine, threonine, histidine and leucine cause considerable reversal of inhibition of *Escherichia coli* by citrinin. The assimilation of leucine, methionine and tyrosine by cells of *Bacillus subtilis* is inhibited by citrinin, the effect being maximum with leucine. The citrinin inhibition of leucine uptake is more with cell-free preparations than with intact cells.**

One approach for studying the mechanism of action of antibiotics is to ascertain metabolites which could reverse inhibition of bacterial cell growth by the specific antibiotic. The antibacterial activity of citrinin is reduced in nutrient broth<sup>1</sup> and Cavallito and Bailey<sup>2</sup> claim complete reversal by cysteine. Studies on the mechanism of action of sulpha drugs have resulted in the recognition of sequential blocking in the bacterial cell of the synthesis of methionine, xanthine, serine, thymine, valine<sup>3-4</sup> and of glycine or threonine<sup>5</sup> in that order. Other examples of reversal of inhibition are of actithiazic acid by biotin and pimelic acid<sup>6</sup> and of aureomycin by riboflavin<sup>7</sup> and by vitamin B<sub>12</sub><sup>8</sup>. This paper relates to a study of metabolites causing reversal of citrinin action in a synthetic medium using *Escherichia coli* Mcleod and high concentrations of citrinin.

The ability of Gram-positive bacteria to assimilate amino acids for protein synthesis<sup>9-10</sup> and interference with this mechanism by penicillin and bacitracin<sup>11</sup> are known. The work of Gale<sup>12-13</sup> reveals that penicillin does not interfere with the passage of glutamate in the cells once the enzymes concerned in this process are elaborated, but interferes with the glutamic acid transporting enzyme. Further work with cell-free preparations<sup>14</sup> established the role of ribonucleic acid (RNA) in this conversion.

Hotchkiss<sup>15-16</sup>, on the other hand, observed that, in presence of penicillin, resting cells formed non-amino nitrogenous material in the surrounding medium indicating that, when penicillin-sensitive component of the cell was inactivated, cellular protein was not formed but there was accumulation of intermediates; Umbreit<sup>17</sup> believes that the component may be a coenzyme of the uridine type.

That penicillin apparently disturbs protein synthesis in Gram-positive

bacteria is evident from the foregoing. Development of resistance to citrinin results in altered amino acid composition of *Bacillus subtilis*<sup>18</sup> indicating a possible interference by citrinin in amino acid metabolism. A study of the influence of citrinin on the amino acid assimilation by *B. subtilis* was therefore undertaken.

## EXPERIMENTAL AND RESULTS

### Antagonism of citrinin action

The growth inhibition of *E. coli* Mcleod by citrinin was studied in salts-synthetic medium of Green and Sevag<sup>19</sup> by the serial dilution method. From turbidity readings on the Klett photo-colorimeter after 20 hr. growth at 30°C., it was observed that citrinin at 300  $\mu\text{g./ml.}$  completely inhibited growth of *E. coli*. Studies on reversal of citrinin action in *E. coli* were therefore carried out in the salts-synthetic medium in presence of 300  $\mu\text{g./ml.}$  citrinin. Results obtained with natural materials and mixtures of vitamins, purines and pyrimidines and of amino acids are recorded in Table 1.

As the reversal of growth inhibition by citrinin obtained with the natural materials could almost completely be duplicated by a mixture of amino acids, the effect of certain individual amino acids was studied (Table 2).

TABLE 1—REVERSAL OF CITRININ ACTION IN *E. COLI*

ADDITIONS TO BASAL MEDIUM	TURBIDITY READINGS AT 660 $m\mu$ .	
	With citrinin	Without citrinin
None	8	62
Peptone		
1.0%	73	105
0.5%	62	82
Yeast extract		
1.0%	96	108
0.5%	64	85
Casein hydrolysate (vitamin-free)		
1.0%	56	69
0.5%	25	62
Vitamin mixture*, 1.0 ml.	37	71
Purine-pyrimidine mixture†, 1.0 ml.	22	65
Amino acid mixture‡, 1.0 ml.	58	96

\* Consisting of: Thiamine HCl, 10  $\mu\text{g.}$ ; nicotinic acid, 2.0  $\mu\text{g.}$ ; Ca-pantothenate, 2.0  $\mu\text{g.}$ ; riboflavin 2.0  $\mu\text{g.}$ ; PABA, 1.0  $\mu\text{g.}$ ; pyridoxamine HCl, 1.0  $\mu\text{g.}$ ; folic acid, 0.3  $\mu\text{g.}$ ; biotin, 0.01  $\mu\text{g.}$ ; pyridoxine HCl, 16.6  $\mu\text{g.}$ ; B<sub>12</sub>, 0.001  $\mu\text{g./ml.}$

† Consisting of 500  $\mu\text{g./ml.}$  each of adenine, guanine, uracil, thymine and xanthine.

‡ Consisting of 80  $\mu\text{g./ml.}$  each of alanine, aspartic acid, glycine, serine, glutamic acid, leucine, isoleucine, lysine, valine, tryptophane, proline, histidine, methionine, threonine, norvaline, arginine, phenylalanine, cystine and cysteine. In the case of optically active amino acids the l-isomer was used.



### Amino acid assimilation

*B. subtilis* was grown in Roux bottles containing 100-ml. lots of peptone water-glucose medium (peptone, 1; NaCl, 0.5; glucose, 1.0 per cent) at pH 7.2. After 20 hr. incubation at 30°C. the pelicle was broken by shaking and cells were separated by centrifugation, washed twice with cold distilled water and made into a homogeneous suspension in cold water to give 30 mg. dry wt. cells per ml.

The incubation mixture used was similar to that of Lester<sup>9</sup> and consisted of 86.5  $\mu$  moles sodium chloride, 160  $\mu$  moles of sodium succinate and the amino acids *l*-leucine, *l*-tyrosine and *dl*-methionine in appropriate concentrations in a total volume of 3.0 ml. Citrinin (1 ml.) in saline citrate was added to the reaction mixture, followed by 1 ml. of cell suspension of *B. subtilis*. After incubation at 37°C. for 2 hr. the cells were spun down in the cold by centrifugation at 18,000  $\times$  g for 10 min. washed once with 5 ml. of ice cold water and recentrifuged. The combined supernatants were steamed for 20 min. and the residual amino acid estimated microbiologically using a cannon-dispenser titration assembly<sup>20</sup>. From 0.04 to 0.2 ml. of the samples were dispensed followed by requisite quantities of distilled water to make 0.2 ml.

TABLE 2—EFFECT OF INDIVIDUAL AMINO ACIDS ON REVERSAL OF CITRININ ACTION IN *E. COLI*

ADDITIONS TO MEDIUM, 0.5 mg./10 ml.	TURBIDITY (24 HR.) AT 660 m $\mu$ .	
	With citrinin	Without citrinin
Control medium	6	58
$\beta$ -Alanine	24	59
<i>dl</i> -Aspartic acid	17	64
<i>l</i> -Arginine	52	75
<i>l</i> (+) Glutamic acid	24	62
Glycine	25	55
<i>l</i> -Histidine	48	62
<i>dl</i> -Isoleucine	22	60
<i>l</i> -Leucine	47	66
<i>l</i> -Lysine	18	54
<i>dl</i> -Methionine	12	56
<i>dl</i> -Norvaline	12	54
<i>l</i> -Proline	24	56
<i>dl</i> - $\beta$ -Phenylalanine	22	58
<i>dl</i> -Serine	18	62
<i>dl</i> -Threonine	43	83
<i>dl</i> -Tryptophan	16	57
<i>dl</i> -Valine	19	60

TABLE 3—INFLUENCE OF CITRININ ON ASSIMILATION OF AMINO ACIDS BY *B. SUBTILIS*

AMINO ACID IN 4 ML. REACTION MIXTURE μg.	CITRININ ADDITION TO 4 ML. REACTION MIXTURE μg.	AMINO ACID ASSI- MILATED PER 30 MG. DRY WT. CELL/2 HR. μg.	INHIBITION %
(Amino acid, <i>l</i> -leucine <sup>21</sup> ; Assay organism, <i>Lactobacillus arabinosus</i> ; Assay range 0-4.8 μg.)			
500	..	139.0	..
1,000	..	98.0	..
1,000	100	60.0	40.8
(Amino acid, <i>l</i> -tyrosine <sup>22</sup> ; Assay organism, <i>Leuconostoc mesenteroides</i> P. 60; Assay range, 0-4.0 μg.)			
500	..	108.0	..
500	100	94.0	12.9
500	200	78.4	27.4
1,000	..	198.5	..
1,000	100	179.0	9.2
1,000	200	126.4	30.8
(Amino acid, <i>dl</i> -methionine <sup>22</sup> ; Assay organism, <i>Lactobacillus fermenti</i> ; Assay range, 0-3.2 μg.)			
500	..	91.7	..
500	100	78.6	13.3
500	200	71.5	20.5
1,000	..	49.0	..
1,000	100	42.0	14.5
1,000	200	38.0	22.5

TABLE 4—INFLUENCE OF CITRININ ON ASSIMILATION OF *l*-LEUCINE BY CELL-FREE EXTRACTS OF *B. SUBTILIS*

ADDITION OF <i>l</i> -LEUCINE TO 4.5 ML. REACTION SYSTEM μg.	CITRININ ADDITION TO 4.5 ML. REAC- TION SYSTEM μg.	<i>l</i> -LEUCINE ASSIMI- LATION PER MG. N. IN 2 HR. μg.	INHIBITION %
1,000	..	140	..
1,000	100	80	42.9
1,000	200	65	53.6

and 0.2 ml. of double strength assay medium. The results are expressed in Table 3.

*l*-Leucine and *dl*-methionine were chosen, the former for its reversal effect and the latter for absence of reversal action (Table 2), and *l*-tyrosine was used as it was present in high concentrations in the resistant strain<sup>18</sup>. Since cell permeability may influence diffusion of amino acids into the cell and its subsequent conversion into cellular proteins, experiments with cell free prepara-

tions of *B. subtilis* were carried out with *l*-leucine only in order to ascertain the influence of citrinin on its uptake.

The cell-free extracts were prepared according to the procedure of McIlwain<sup>23</sup>. The extract (1.5 ml.) obtained from 8 g. wet wt. cells in 20 ml. was added to the reaction system as above and incubated at 37°C. for 2 hr., the reaction was then stopped by 0.5 ml. of 0.6 N. sulphuric acid followed by 0.4 ml. 10 per cent sodium tungstate according to the method of Soloman *et al*<sup>24</sup>. After removing the precipitate by centrifugation, the supernatant was steamed, neutralized with 3 N caustic soda and made to volume. The residual free leucine was estimated microbiologically.

The nitrogen content of the cell-free extracts was determined by a micro method by direct nesslerization after digestion with phosphoric acid-sulphuric acid copper sulphate reagent (Table 4).

With 1,000  $\mu$ g. of *l*-leucine and using 1,000  $\mu$ g. adenylic acid as phosphate acceptor it was observed that, in control system (without added citrinin), *l*-leucine uptake/mg. nitrogen 2 hr. was increased by 9 per cent. When 2:4 dinitrophenol was added in a concentration of 200  $\mu$ g. (comparable to the amounts used to uncouple phosphorylation from oxidation)<sup>25</sup>, it was observed that this compound was inhibitory to the growth of the assay organism and hence its effect could not be ascertained.

## DISCUSSION

Citrinin action in *E. coli* is readily reversed by natural materials like peptone and yeast extract the effect being almost duplicated with a mixture of amino acids and not with mixtures of B-vitamins, or purines and pyrimidines (Tables 1) indicating a possible interference by citrinin in amino acid metabolism of the organism.

Although individual amino acids caused growth reversal to different degrees, arginine, histidine, leucine and threonine caused good reversal indicating that citrinin may be interfering in particular with the utilization of these amino acids.

The cells of *B. subtilis* take up leucine at a faster rate than either methionine or tyrosine at lower concentrations of these amino acids. At higher levels of these amino acids, the disappearance of leucine and methionine is at a slower rate when compared to that of tyrosine. Citrinin inhibits considerably the uptake of all three amino acids studied, the effect being more with leucine.

Cell-free extracts take up leucine at a considerable rate. Adenylic acid (Adenosine-5-phosphoric acid) slightly stimulates the disappearance of leucine suggesting the introduction of more favourable condition for amino acid uptake. The greater inhibition of leucine uptake with cell-free extracts would indicate that citrinin inhibits the leucine transporting system of *B. subtilis*.

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## Discussion

DR. J. N. TAYAL: Are the results in respect of ribose nucleic acid content in normal and resistant organisms averages of several results? Has the significance of variations in the results been assessed?

DR. D. V. TAMHANE: Values given are averages of at least three determinations from independent experiments and only significant differences have been commented on.

# Influence of Some Antibiotics on the Biosynthesis of Ascorbic Acid

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The influence of penicillin, streptomycin, aureomycin and terramycin in germinating green gram seedlings has been studied. Aureomycin and terramycin have marked inhibitory effect on chlorophyll formation. Aureomycin and terramycin have also been shown to decrease ascorbic acid content while, at the same time, increasing the ratio of dehydro-ascorbic acid to ascorbic acid. The significance of this increase in dehydro-ascorbic acid to ascorbic acid has been discussed. Aureomycin and terramycin have no effect on the utilization of glucurone for ascorbic acid synthesis. Penicillin and streptomycin, at the levels employed, have been found to have no influence on any of the systems studied.

During the past few years, considerable amount of work has been done towards understanding the mode of action of antibiotics which shows that antibiotics affect various enzyme systems and reactions. Most of the work has been carried out either in bacteria or in *in vitro* systems using mitochondria. From such studies, evidence has been forthcoming that penicillin inhibits protein synthesis<sup>1</sup> and causes a profound disturbance of nucleic acid metabolism<sup>2</sup>, but does not affect carbohydrate metabolism. Oginsky *et al.*<sup>3</sup> have shown that streptomycin inhibits an oxaloacetate-pyruvate condensation, and hence the citric acid cycle in *Escherichia coli*. Loomis<sup>4</sup> has shown that aureomycin acts as an uncoupler of oxidative phosphorylation, and Osteux *et al.*<sup>5</sup> have found that both aureomycin and terramycin inhibit the operation of the citric acid cycle. Dudani and Krishnamurti<sup>6</sup> have recently found that terramycin has a specific oxidative action on ascorbic acid.

Little work has been done with antibiotics in plant systems, and in view of some of the experimental findings mentioned above, it was considered worthwhile to study whether penicillin, streptomycin, aureomycin and terramycin had any effect on ascorbic acid synthesis or metabolism in germinated seedlings.

## EXPERIMENTAL

Green gram (*Phaseolus radiatus*) seeds were used in the studies. Two grams of healthy, green gram seeds were germinated for 72 hr. in 9 cm. Petri

dishes on discs of filter paper. The substances whose effects were to be studied were added in 10 ml. of medium in all the cases. The antibiotics were dissolved in sterile water and the solution then pipetted aseptically into the respective Petri dishes. Glucurone was added 24 hr. after the beginning of germination as there was about 10-20 per cent inhibition of germination if it was added at the commencement itself. All germinations were carried out according to the procedure described earlier<sup>8,7</sup>.

After germination, the seedlings were rinsed with distilled water, ground up with metaphosphoric acid, centrifuged, and made up to volume, the final concentration of metaphosphoric acid being 4 per cent. An aliquot was taken and its dehydroascorbic acid content (DHA) estimated. Another aliquot was shaken up with a few drops of bromine, the excess bromine removed by aeration and total ascorbic acid content (expressed as vitamin C) determined. All estimations were carried out according to the method of Roe and Kuether<sup>9</sup>, using 2,4-dinitrophenylhydrazine, the colour developed being compared against blank, in a Lumetron colorimeter at 530 m $\mu$ .

### RESULTS AND DISCUSSION

Various levels of penicillin, streptomycin, aureomycin, and terramycin were tried, the level chosen being, in each case, the highest level which did not inhibit growth appreciably. The influence of these antibiotics on the growth and metabolism of the germinating seedlings is summarized in Table 1. The numerical figures given, apart from the levels of the antibiotics, are approximate and represent visual estimates.

The levels of antibiotics given in Table 1 were used for further investigations. At higher levels, all four antibiotics tended to be inhibitory towards germination and growth, and hence the effects of such levels on ascorbic acid synthesis have not been studied. It is of interest to note that neither penicillin which inhibits protein synthesis<sup>1</sup> nor streptomycin which inhibits the oxalo-

TABLE 1—INFLUENCE OF SOME ANTIBIOTICS ON GERMINATING SEEDLINGS

	ANTIBIOTIC LEVEL mg./10 ml.	INHIBITION OF GROWTH %	DECREASE IN CHLOROPHYLL FORMATION %	EFFECT ON	
				Root growth	Vitamin C content*
Terramycin	3.0	10	70-80	Short, stubby roots	Decreased
Aureomycin	7.5	10	70-80	do.	Decreased
Penicillin	10.0	0	0	Normal	†
Streptomycin	15.0	0	0	Short, stubby roots	†

\* Detailed values are given in Tables 2 and 3.

† Value same as in control.



acetate-pyruvate condensation<sup>3</sup> affect vitamin C levels. This is in keeping with the prevailing concepts of biosynthesis of ascorbic acid.

Aureomycin and terramycin were markedly inhibitory, as far as growth was concerned, above the levels stated in Table 1. At the levels tried, however, there was only slight inhibition of growth as seen mainly in root growth, but a considerable inhibition of chlorophyll formation was observed, the seedlings closely resembling those grown in darkness. No definite explanation can be given for the effect, but probably this is due to interference with magnesium utilization, as the recent work of Brody *et al.*<sup>10</sup> would suggest.

The influence of aureomycin and terramycin on ascorbic acid (AA), dehydro-ascorbic acid (DHA), and total ascorbic acid (vitamin C) content has been given in Tables 2 and 3.

It can be seen that both aureomycin and terramycin have a similar influence on the levels of DHA, AA, and the ratio DHA/AA. There is a decrease in vitamin C level. However, the amount of DHA does not fall. On the contrary, the DHA/AA ratio shows an increase (Tables 2 and 3). This trend towards a higher value than the control in presence of aureomycin or terramycin is significant. It should be remembered that ascorbic acid by virtue of its capacity to readily undergo enzymatic oxidation and reduction in plant tissues has an important role as a respiratory carrier as has been suggested earlier. This functional role of ascorbic acid has been discussed by Mapson<sup>11</sup>

TABLE 2—EFFECT OF AUREOMYCIN ON ASCORBIC ACID SYNTHESIS

(Values in mg./100 g. seeds)

SUBSTANCES IN CULTURE MEDIUM (per 10 ml.)	VITAMIN C		DHA	AA	DHA/AA %
	Total	Difference			
Water only (control)	120	100	25	95	26.4
Glucurone (50 mg.)	220		30	190	15.8
Aureomycin (7.5 mg.)	105	85	25	80	31.2
Aureomycin (7.5 mg.) + Glucurone (50 mg.)	190		40	130	26.6

TABLE 3—EFFECT OF TERRAMYCIN ON ASCORBIC ACID SYNTHESIS

(Values in mg./100 g. seeds)

SUBSTANCES IN CULTURE MEDIUM (per 10 ml.)	VITAMIN C		DHA	AA	DHA/AA %
	Total	Difference			
Water only (control)	125	95	20	105	19.0
Glucurone (50 mg.)	220		22.5	197.5	11.4
Terramycin (3 mg.)	105	95	18.5	86.5	21.4
Terramycin (3 mg.) + Glucurone (50 mg.)	200		25	175	14.3

recently, who states that "what is even more important than the level of AA is the concentration of DHA, which by reason of its ability to accept hydrogen may determine the level at which the ascorbic acid system acts as a respiratory catalyst". The results presented in Tables 2 and 3 suggest, therefore, that this system may be acting in each case at a higher level than normal, in presence of the antibiotic. The action of the tetracycline antibiotics as uncouplers of oxidative phosphorylation would necessitate an increased respiratory rate in view of the decreased energy availability from carbohydrate oxidation under these conditions, and this increase of DHA/AA ratio would then reflect the reaction of the plant respiratory systems. The decreased synthesis of chlorophyll observed also points in the same direction, since Brody *et al.*<sup>10</sup> have shown that uncoupling of oxidative phosphorylation by tetracyclines in mitochondrial preparations is pronounced at low  $Mg^{++}$  concentrations and becomes negligible at high  $Mg^{++}$  concentrations.

The decrease in ascorbic acid levels induced by these antibiotics, would also be attributable to increased substrate oxidation in the germinating seedlings, since ascorbic acid is known to be metabolized in a similar manner to carbohydrates, as shown by the work of Burns, Burch and King<sup>12</sup>.

The utilization of glucurone, which is an established and important precursor of ascorbic acid<sup>13,14</sup> in the sequence:

*d*-Glucose  $\rightarrow$  *d*-Glucurone  $\rightarrow$  *l*-Gulono-lactone  $\rightarrow$  *l*-Ascorbic acid is slightly diminished by aureomycin and is unaffected by terramycin (Tables 2 and 3). It cannot, however, be stated to be definitely due to an adverse influence of aureomycin on the utilization of glucurone.

Tables 2 and 3 also show that in presence of glucurone, there is an increase in AA, due to biosynthesis from the added intermediate, and at the same time the DHA/AA ratio decreases. This is to be expected, if in the biosynthetic sequence leading from glucurone to ascorbic acid, ascorbic acid is first produced, this being converted to dehydroascorbic acid by the plant systems as and when required.

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### Discussion

DR. P. N. NANDI: Is the inhibitory effect of antibiotics on chlorophyll formation reversible?

DR. S. K. BOSE: Some intermediate products in the biosynthesis of ascorbic acid have already been characterized. What is the influence of these antibiotics on the above biochemical intermediates?

DR. P. S. SARMA: Only glucurone has been studied and no other intermediates ; whether the inhibition of chlorophyll formation is reversible has not been studied.



# Effect of Penicillin and Aureomycin on Enzyme Production by *Vibrio cholerae*

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The effect of penicillin and chlorotetracycline (aureomycin) incorporation in the growth medium of *V. cholerae* on the metabolic activity of the organism as represented by a few typical enzymes has been studied. Representative cultures of *V. cholerae* were grown in media containing penicillin and aureomycin and the cells were harvested. Penicillin and chlorotetracycline were found to have a stimulatory action on the enzymes, aspartic, adenosine, and serine deaminases, lactic dehydrogenase, tryptophanase, etc. except in the case of aspartic deaminase which was stimulated in the Ogawa subtypes and not appreciably affected in the Inaba subtypes.

Development of drug resistance by a micro-organism is accompanied by striking changes in its metabolic activity<sup>1</sup>. Acquisition of resistance to streptomycin thus results in an increase in the ribonucleic acid content of bacterial cells<sup>2</sup>. A similar increase in the intracellular concentration of *p*-aminobenzoic acid has been reported in sulphanilamide resistant bacteria<sup>3</sup>. Involvement of enzymes in the mechanism of drug resistance is evident from the well-known examples of penicillinase synthesis in penicillin resistant bacteria or the production of organic nitro reductase in resistance to chloramphenicol<sup>4-5</sup>. Development of alternate pathways particularly in the terminal respiratory cycle is another instance of enzyme variation resulting from drug resistance<sup>6</sup>. Analysis of such enzyme variations has proved to be invaluable in elucidating the mechanism of drug resistance<sup>7-8</sup>.

The observations reported in this paper arose out of a study of the sensitivity of *Vibrio cholerae* to different antibiotics. Several strains of the organism were found to grow, albeit at a reduced rate, in presence of bacteriostatic concentrations of chlorotetracycline. Although *V. cholerae* is not sensitive to penicillin it undergoes striking morphological variations<sup>9</sup> in presence of the drug and whether these changes are reflected in the metabolic activity of the organism is not clear. In view of these considerations and as part of an investigation on the factors affecting the enzyme activity of *V. cholerae*, it was of interest to examine the effect of penicillin and chlorotetracycline incorporation in the growth medium of *V. cholerae* on the general metabolic activity of the organism as represented by a few typical enzymes.

TABLE 1—EFFECT OF PENICILLIN INCORPORATION ON *VIBRIO CHOLERAE* ENZYMES

CULTURES	DEAMINASES ( $\mu$ g ammonia liberated in 60 min.)										DEHYDROGENASE (Time for discharging colour of methylene blue, substrate, lactate, sec.)	
	Aspartate		Adenosine		Serine		Penicillin		Penicillin		Control	Penicillin
	Control	Penicillin	Control	Penicillin	Control	Penicillin	Control	Penicillin				
Og D25/53	14.0	16.0	32.0	37.0	7.0	8.5	420	265				
Og 52	26.0	34.0	20.0	28.0	6.0	7.0	480	360				
Og 132	22.0	29.0	18.0	24.0	8.0	8.5	605	300				
In 71/53	14.0	14.5	18.0	22.0	6.0	6.5	960	510				
In 49514	20.0	20.0	21.0	41.0	7.0	8.2	335	255				
Water Khal	18.5	26.0	38.0	43.0	8.5	11.5	285	165				
NAG 2	49.0	53.0	37.0	47.0	25.0	30.0	225	160				
Rough 569 B	20.0	20.0	—	—	30.5	39.5	265	205				

## EXPERIMENTAL

Representative cultures of the Ogawa and Inaba sub-types, rough variants and non-choleraenic water vibrios were included in this study. The organism was adapted to grow by successive transfers on papain meat agar containing either 5 units of penicillin per cc. or 25  $\mu$ g. of chlorotetracycline per tube. Growth in presence of penicillin was comparable to that on the un-supplemented medium. Growth on chlorotetracycline supplemented medium was poor and adequate quantities of cells for enzyme studies could be obtained only after 48 hr. incubation. The cell suspension for enzymatic studies was made by harvesting the growth with saline, washing, centrifugation and adjustment of opalescence turbidimetrically to a standard value<sup>10</sup>. Deamination of aspartate, adenosine and serine was followed by estimating the ammonia liberated<sup>11</sup>. Oxygen uptake and dehydrogenase activities were measured by conventional manometric and Thunberg techniques. Indole production from tryptophane was followed up colorimetrically<sup>12</sup>.

TABLE 2—EFFECT OF PENICILLIN ON TRYPTOPHANASE OF VIBRIO CHOLERAEE

INDOLE FORMED FROM TRYPTOPHANE IN 60 MIN. ( $\mu$ g)

	INDOLE FORMED FROM TRYPTOPHANE IN 60 MIN. ( $\mu$ g)	
	Control	Penicillin
Ogawa D25/53	5.0	17.0
Inaba 71/53	5.1	12.0
Inaba 49514	9.5	14.0
Inaba 49524	16.7	19.8
Water Khal	9.5	11.8

TABLE 3—EFFECT OF PENICILLIN ON RESPIRATION OF VIBRIO CHOLERAEE

OXYGEN UPTAKE IN 60 MIN. ( $\mu$ l.)

	Glucose		Pyruvate	
	Control	Penicillin	Control	Penicillin
Ogawa 123	21.0	31.0	26.0	45.0
Inaba 123	13.5	24.0	18.0	32.0
Ogawa D25/53	—	—	26.0	45.4
Inaba 113/53	—	—	35.7	43.0
Inaba 49514	—	—	52.0	68.0
Inaba 49524	—	—	54.0	66.0
Ogawa M55/56	21.0	31.0	—	—
Inaba M71/53	13.0	24.0	—	—
Water Khal	35.0	50.0	70.0	76.0
NAG 6	28.0	36.0	41.8	56.0



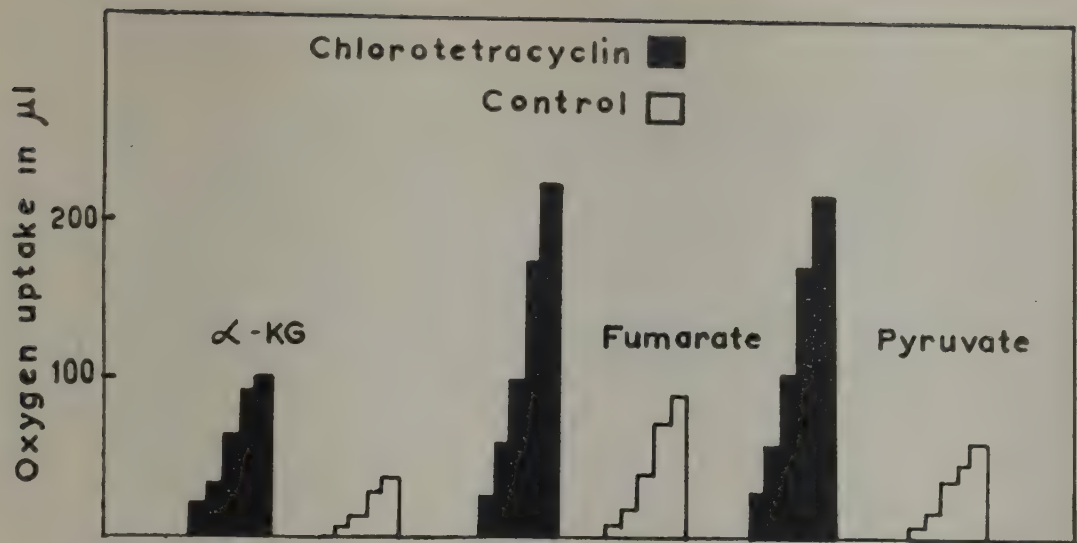


FIG. 1—EFFECT ON OXIDATION OF  $\alpha$ -KETOGLOUTARATE, FUMARATE AND PYRUVATE BY *V. cholerae* CELLS GROWN IN PRESENCE OF CHLOROTETRACYCLIN

### RESULTS

#### Effect of Penicillin

The effect of incorporating penicillin in the growth medium on aspartic acid, adenosine and serine deaminases, lactic dehydrogenase, tryptophanase and oxygen uptake in presence of glucose and pyruvate is brought out in Tables 1-3. It is apparent that penicillin has a stimulatory action on all these enzymes with practically all the strains studied. The only exception is aspartic acid deaminase which is found to be stimulated by the drug in the Ogawa subtypes and not appreciably affected in the Inaba subtypes as brought out in Table 4.

TABLE 4—EFFECT OF PENICILLIN ON ASPARTIC ACID DEAMINASE OF OGAWA AND INABA SUBTYPES

	ASPARTIC ACID DEAMINASE: $\mu$ g AMMONIA RELEASED IN 60 MIN.	
	Control	Penicillin
52 Ogawa	26.0	34.0
132 Ogawa	22.0	29.0
558 Ogawa	22.5	27.0
428 Ogawa	36.0	42.5
D25/53 Ogawa	14.0	16.0
52 Inaba	19.0	17.5
132 Inaba	34.0	35.0
341 Inaba	40.5	37.0
49514 Inaba	20.0	19.5
49524 Inaba	20.0	19.5
123 Inaba	36.5	35.0

TABLE 5—EFFECT OF CHLOROTETRACYCLINE INCORPORATION ON V. CHOLERAE ENZYMES

CULTURES	DEAMINASES ( $\mu$ g. ammonia liberated in 60 min.)										TRYPTOPHANASE ( $\mu$ g. indole formed in 60 min.)	
	Aspartate		Adenosine		Serine							
	Chlorotetracycline		Chlorotetracycline		Chlorotetracycline		Chlorotetracycline		Chlorotetracycline		Chlorotetracycline	
	Control	Chlorotetracycline	Control	Chlorotetracycline	Control	Chlorotetracycline	Control	Chlorotetracycline	Control	Chlorotetracycline	Control	Chlorotetracycline
Og D 25/53	15.0	21.0	32.0	35.0	1.5	4.0	—	—	2.1	6.5	—	—
Og 49518	11.0	16.5	17.5	8.0	7.0	13.5	—	—	5.3	6.2	—	—
Og 49514	5.5	9.5	8.5	9.0	5.5	7.5	—	—	4.0	7.1	—	—
Og 54/53	—	—	—	—	—	—	—	—	3.2	4.8	—	—
Og 52	—	—	—	—	—	—	—	—	2.8	6.5	—	—
In 52	7.0	15.5	10.0	13.0	—	—	—	—	3.7	6.5	—	—
In 49514	19.0	21.5	21.5	38.5	—	—	—	—	4.2	8.0	—	—
In 49524	36.5	41.0	23.0	34.0	—	—	—	—	9.8	12.4	—	—
In 113/53	—	—	—	—	—	—	—	—	4.0	8.0	—	—
In 117/53	—	—	—	—	—	—	—	—	12.4	16.0	—	—
R. 49514	9.0	21.0	12.0	20.0	5.0	10.0	—	—	8.8	9.4	—	—
Kimboghath	12.0	20.0	22.0	32.0	—	—	—	—	—	—	—	—
Water Khal	8.0	22.0	8.0	40.0	5.0	10.0	—	—	11.6	16.0	—	—
NAG 6	10.0	15.0	21.0	36.0	—	—	—	—	—	—	—	—
Water tank	5.0	15.0	5.0	15.0	5.0	9.0	—	—	1.6	4.4	—	—
NAG 1	—	—	—	—	—	—	—	—	11.6	14.2	—	—

### Effect of chlorotetracycline

The effect of chlorotetracycline on the corresponding enzymes is presented in Tables 5 & 6. Fig. 1 represents the oxygen uptake of the control and chlorotetracycline supplemented cells in presence of  $\alpha$ -ketoglutarate, fumarate and pyruvate. It would be clear that the general pattern of results is similar to that observed with penicillin. The difference noticed in the behaviour of aspartic acid deaminase of the Ogawa and Inaba subtypes in presence of penicillin is not observed with chlorotetracycline. It is also clear that greater stimulation is brought about by chlorotetracycline in practically all the enzymes.

### DISCUSSION

The stimulatory effect of penicillin and chlorotetracycline incorporation on the enzymes studied was observed during successive transfers and would thus appear to be a genuine irreversible stimulation of activity. Since the stimulation is present with practically all the enzyme systems studied it would appear that the drugs raise the overall metabolic level of the cells. An abnormal metabolism has been reported in *Neurospora* in presence of toxic concentrations of *p*-amino benzoic acid<sup>13</sup>. Weight to weight, no significant increase in nitrogen content of cells was observed on addition of the antibiotics excluding thereby possibility of synthesis of larger amounts of proteins under the stress of the drugs. Apparently the reserve protein of the cells is adapting itself to function as enzymes when stimulated by the drugs. An analogous phenomenon is observed in induced enzyme synthesis in bacteria in the resting phase in the absence of a nitrogen source but in contact with the inductive agent<sup>14</sup>.

The results reported here raise various interesting questions. Is the variation in enzyme activity a genotypic change or a phenotypic one? How far stable are these changes on bringing back the cells to the normal medium? Will the stimulation of enzyme activity by the drugs result in alterations in pathogenicity and virulence? While answers to these questions can be pro-

TABLE 6—EFFECT OF CHLOROTETRACYCLINE ON RESPIRATION OF *VIBRIO CHOLERA*E  
(Oxygen uptake in 60 min.)

	GLUCOSE		PYRUVATE	
	Control	Chloro.	Control	Chloro.
Ogawa D25/53	—	—	26.0	89.0
Inaba 113/53	—	—	35.0	88.0
Inaba 49514	—	—	12.0	44.0
Inaba 49524	—	—	16.0	54.0
NAG 6	20.0	48.0	72.0	86.0
Water Khal	28.0	44.0	90.0	118.0



vided only after further work, the observations reported indicate that quantitative changes in the normal cellular enzymes may be of some significance in the development of drug resistance by *V. cholerae*.

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### Discussion

DR. S. K. BOSE: Were the kinetics of stimulation process of the enzymes studied?

DR. C. R. KRISHNA MURTI: No.

DR. V. N. KRISHNAMURTHY: Is there any increase of R.D.E. and mucinase produced by penicillin and tetracycline when added to cholera culture?

DR. C. R. KRISHNA MURTI: The work is being continued by my colleagues and will be reported on later.

# Action of Antibiotics on Enzyme Systems

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The effect of penicillin, dihydrostreptomycin, chloramphenicol, chloro- and oxytetracyclines has been studied on enzyme systems of tissues and pathogens like *Vibrio cholerae* and *Salmonella typhosa*. The enzyme systems examined include those dependent on active thiol or metal groups and specific coenzymes like pyridoxal phosphate, flavin adenine nucleotide or coenzyme A.

With the exception of penicillin, the action of the other antibiotics on the enzymes studied was widespread and non-specific. The degree of inhibition was found to depend upon the time of contact between enzyme and antibiotic and the concentration of antibiotic and substrate. In a number of enzymes, inhibition was found to be of the reversible and competitive type with respect to substrate.

Enzyme inhibition studies have greatly helped in the elucidation of the specificity of action of antibacterial agents in inhibiting the growth of micro-organisms without appreciable damage to the host<sup>1,2</sup>. There is also increasing evidence to show that the vital problem of drug resistance is in one way or the other associated with alterations in the enzyme pattern of microbes<sup>3-4</sup>. In view of these considerations, investigations on the effects of antibiotics on the diverse metabolic activities of tissues and bacteria have been in progress in the Institute, for the last few years and a preliminary account of the observations was presented earlier<sup>5</sup>. The purpose of this paper is to report on the progress made subsequently in this direction and summarize the main findings reached so far.

## EXPERIMENTAL

Pure crystalline samples of penicillin G (Indian Penicillin Committee), dihydrostreptomycin sulphate (Glaxo Laboratories), chloramphenicol (chloromycetin, Parke Davis & Co.), chlorotetracycline (aureomycin hydrochloride, Lederle Laboratories) and oxytetracycline (terramycin hydrochloride, Charles Pfizer & Co.) were used in these studies.

The catalase used was a partially purified preparation from goat liver<sup>6</sup> and was assayed by the permanganate titration of unreacted hydrogen peroxide<sup>7</sup>. Taka diastase (Parke Davis & Co.) and saliva of locusts served as sources for aryl-sulphatase which was assayed by the colorimetric estimation of nitro-catechol released by the hydrolytic fission of K-2-hydroxy-5-nitro-phenyl sulphate<sup>8</sup>.

A cell-free extract of *Vibrio cholerae* was used as source of organic nitro reductase and deaminases. Activity of nitro reductase was followed by the photometric estimation of the arylamine formed<sup>9</sup>. An active preparation of *d*-amino acid oxidase from pig kidney<sup>10</sup>, notatin<sup>11</sup> isolated from the fermentation broth of *Penicillium notatum* Westling and xanthine oxidase of milk cream<sup>12</sup> and rat liver<sup>13</sup> were used as representatives of the flavin group of enzymes and activity was followed by conventional manometric methods or anaerobic reduction of methylene blue.

Other enzyme preparations, their methods of assay and relevant experimental details adopted in the study of inhibition have been referred to in earlier publications<sup>14-21</sup>. Briefly, the method consisted in incubating the enzyme and antibiotic in appropriate concentrations in a buffered system and assaying the activity after a fixed interval. Enzyme buffer mixture with water was always included as a control. Reversal of inhibition was studied by adding the appropriate reversing agent after exposing the enzyme to drug action for a definite period.

## RESULTS AND DISCUSSION

The results obtained are summarized below.

### SH-Enzymes

Chloramphenicol, dihydrostreptomycin and the tetracyclines inhibited urease—Reactivation was brought about by glutathione or hydrogen sulphide. Penicillin had a slight activating action on the enzyme. The proteinase activity of papain was accelerated by all the antibiotics whereas the chymase activity was inhibited almost completely although the activity could be restored by passing hydrogen sulphide. Action of these antibiotics on amylases of different sources was not appreciable excepting a partial inhibition of bacterial amylase brought about by chloramphenicol.

### Metal complex enzymes

Dihydrostreptomycin and oxytetracycline were inhibitory to alkaline phosphatase of rat kidney. Chlorotetracycline had a slight activating action on the rat kidney enzyme but a significant inhibitory effect on the phosphatase activity of resting cells of *Salmonella typhosa*<sup>22</sup>. The inhibition observed was readily reversed by magnesium salts. Inhibition of rat kidney phosphatase by oxytetracycline was competitive with respect to substrate. All the antibiotics had depressing action on arginase activity of liver homogenates. The inhibition was competitive and reversible by manganese salts. None of the antibiotics tried had any appreciable affect on organic nitro reductase in contrast to the reported inhibitory action of chlorotetracycline on the nitro reductase of *Escherichia coli*<sup>23</sup>.

### Enzymes involved in oxidative metabolism of tissues and bacteria

Oxytetracycline was found to affect uniformly both the dehydrogenase and cytochrome oxidase of the succinic oxidase complex of pigeon breast muscle



and pig heart. Excess of cytochrome, cysteine or succinate had no protective action. The partial inhibitory effect of dihydrostreptomycin on the dehydrogenase was, however, reversed by cysteine. All the drugs excepting penicillin had significant inhibitory action on the oxygen consumption of *V. cholerae* cells in presence of Kreb's cycle intermediates and the amino acid feeders of this cycle whereas they were without any effect whatsoever on the corresponding metabolic activity of rat liver homogenate. A coenzyme preparation made from rabbit liver protected the cells partially from the deleterious action of chloramphenicol but not of the tetracyclines. The drugs studied had no effect on the flavin adenine enzymes. The tetracyclines were found to have adverse action on goat liver catalase.

### Enzymes involved in intermediary metabolism of amino acids and purine pyrimidine compounds

Indole production from tryptophane by resting cells of *E. coli* was considerably inhibited by chloro- and oxytetracyclines. The inhibition was of the competitive type with respect to substrate but irreversible by yeast extract. None of the antibiotics tried had any action on the aspartic acid deaminase of a cell-free extract of *V. cholerae*. All drugs excepting penicillin had, however, some inhibitory action on the adenosine and cytidine deaminases of the same extract.

It would be seen from these results that the action of the antibiotics tried with the exception of penicillin was rather widespread and apparently non-specific. The degree of inhibition was found to vary with the time of contact between enzyme and antibiotic indicating a stoichiometric association of the drug and the inhibitor. The concentrations of substrate and antibiotic used in many instances were also found to influence the degree of inhibition suggesting a competitive type of action between inhibitor and substrate for attachment to the active centres of the enzyme.

The lowering of enzyme activity observed in many instances could be arrested by the addition of appropriate protective agents. In this respect, the reversal of the inhibition of thiol dependent enzymes by thiol compounds and the metal dependent enzymes by metallic ions was complete. These observations lend support to the view that the combination of antibiotic with active thiol groups or metal moieties of the enzyme molecule may play an important part in their antibacterial action. The inhibitory action of antibiotics observed on enzymes dependent on specific coenzymes does not appear to involve a direct inactivation of these factors since even excess of these cofactors had no protective action of the enzymes inhibited. This can be explained on the basis of partial or complete denaturation of the protein moiety of the enzyme under the influence of the drugs.

In general, the action of the antibiotics on the enzymes studied has been found to be of a diverse character and possibly non-specific in nature. This is not difficult to understand in view of the wide differences in the chemical structure of these drugs. The presence of the tetracycline ring in both chloro- and oxytetracyclines would be expected to confer on these similar properties.

Many instances have, however, been observed during the course of this study indicating rather wide diversity of action between these two drugs. Oxytetracycline inhibits the alkaline phosphatase of rat tissues and has no action on the corresponding enzymatic activity of *S. typhosa*, whereas chlorotetracycline has no action on the former but inhibits the latter. Again oxytetracycline brings about an irreversible inhibition of the succinic oxidase system and catalyses the auto-oxidation of ascorbic acid<sup>24</sup>, chlorotetracycline does not exhibit these properties. On the other side, a number of reactions like the deamidasic hydrolysis of arginine and urea, indole production from tryptophane, oxidation of tricarboxylic acid cycle intermediates by bacterial cells and the deamination of adenosine and cytidine and catalase are inhibited by both the drugs.

A rather significant observation made in the course of this study is the specific inhibitory effect of the tetracyclines on the terminal respiration of bacterial cells without affecting the corresponding metabolic activity of rat liver homogenates. The nature of this inhibition needs further elucidation by detailed studies on the isolated enzyme systems which mediate the tricarboxylic acid cycle. The results obtained thus far permit us to conclude that the therapeutic effectiveness of these drugs may be the result of the blocking of a whole cycle of reactions rather than due to the inactivation of a single enzyme. The anabolic processes within the cell are the outcome of the energy released during the terminal oxidation of primary metabolites and the extension of inhibition studies to enzymatic reactions involved in the synthetic processes of the bacterial cell may reveal many interesting facts concerning the mode of action of antibiotics.

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# Tetracycline Antibiotics and Nucleotide Metabolism in Micro-organisms

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The inter-relationship between the tetracycline antibiotics on the one hand and the vitamins, folic acid and B<sub>12</sub>, on the other in respect of nucleotide metabolism in micro-organisms has been discussed.

Aureomycin inhibits the synthesis of both ribose- and desoxy-ribose nucleic acids. Folic acid and to a lesser extent, vitamin B<sub>12</sub> exert protection against this action.

Aureomycin and terramycin both inhibit the enzymic conversion of uracil to thymine by respiring cells of *Bacillus subtilis*.

The bacteriostatic action of penicillin has been demonstrated to be primarily due to a disturbance in nucleic acid metabolism<sup>1</sup>. The tetracycline antibiotics, like penicillin, exert their antibacterial action, especially in the early stage of bacterial growth<sup>2</sup>, which is associated with active nucleic acid synthesis. Since folic acid (PGA) and vitamin B<sub>12</sub> are known<sup>3</sup> to mediate nucleic acid synthesis in micro-organisms it was of interest to ascertain the relationship in this respect between these vitamins and the antibiotics.

The presence of an enzyme system capable of converting uracil to thymine in cells of *Bacillus subtilis* was demonstrated in this laboratory<sup>4</sup>; both folic acid and vitamin B<sub>12</sub> were shown to influence this reaction. A study of the effect of the antibiotics on this reaction was included.

## EXPERIMENTAL AND RESULTS

### Nucleic acid synthesis

Much work has been done on the influence of PGA and vitamin B<sub>12</sub> on nucleic acid metabolism with *Lactobacillus casei*<sup>3,5</sup>. *L. casei* (ATCC 7469) was used in these studies on nucleic acid synthesis. Methods of culturing, harvesting and nucleic acid determinations were those described previously<sup>3</sup>. A concentration of aureomycin (crystalline, Lederle) was chosen which produced half maximum growth inhibition in tube assays and was added to the previously sterilized medium after Seitz filtration. A 48-hr. harvest was used for the study as growth was delayed in presence of the antibiotic. Average results of duplicate experiments are given in Table 1.

TABLE 1—AUREOMYCIN INTERFERENCE WITH NUCLEIC ACID SYNTHESIS IN *L. CASEI*

ADDITIONS PER 100 ML. BASAL MEDIUM	WITHOUT AUREOMYCIN			WITH AUREOMYCIN 300 M $\mu$ G/100 ML.		
	Cell mass (g./l.)	RNA (%)	DNA (%)	Cell mass (g./l.)	RNA (%)	DNA (%)
Folic acid, 200 m $\mu$ g.	0.54	11.32	1.92	0.48	9.56	1.67
B <sub>12</sub> , 200 m $\mu$ g.	0.18	8.80	1.85	0.16	7.28	0.83
Folic acid + B <sub>12</sub> , 200 m $\mu$ g. each	0.60	10.00	2.80	0.46	9.05	2.54
B <sub>12</sub> , 400 m $\mu$ g.	0.33	12.42	2.14	0.27	12.17	2.08
Folic acid, 200 m $\mu$ g. + B <sub>12</sub> , 400 m $\mu$ g.	0.65	11.67	3.85	0.60	12.14	2.78

TABLE 2—CONVERSION OF URACIL TO THYMINE BY *B. SUBTILIS* INTERFERENCE  
BY TETRACYCLINE ANTIBIOTICS

ANTIBIOTIC	THYMINE FORMED IN 4 HR. ( $\mu$ g.)			INHIBITION (%)
	10 mg. cells	10 mg. cells + 1 mg. uracil	Net	
Nil	14.2	124.6	110.4	—
Terramycin, 10 $\mu$ g.	13.8	86.9	73.1	34
Terramycin, 100 $\mu$ g.	13.4	48.2	34.8	69
Aureomycin, 10 $\mu$ g.	13.5	88.2	74.7	32
Aureomycin, 100 $\mu$ g.	12.9	51.7	38.8	65

### Conversion of uracil to thymine

The organism employed was *B. subtilis*, a local isolate, grown in the salts-dextrose medium of Green and Sevag<sup>6</sup>. Experimental details were those described previously<sup>4</sup>. The results using both aureomycin and tetracycline each at two different levels are given in Table 2.

### CONCLUSION

Aureomycin inhibits the synthesis of both ribose- and desoxy ribose-nucleic acids (RNA & DNA). PGA exerts protection against this action. A higher concentration of vitamin B<sub>12</sub> is required to provide similar adequate protection. The decrease in RNA in presence of aureomycin is seen to be more when the decreased cell mass is taken into consideration. It also seems that the impairment in RNA synthesis is far greater than the extent of inhibition of growth as expressed in terms of cell mass. Apparently, the decreased RNA reflects in diminished synthesis of the cellular proteins<sup>7</sup>. Smolens and Vogt<sup>8</sup> have observed that cultural conditions favouring increased RNA content of bacterial cells enhance resistance of *Hemophilus pertussis* to aureomycin among other antibiotics,

The biosynthesis of thymine from uracil in *B. subtilis* is inhibited by both aureomycin and terramycin. Since PGA and vitamin B<sub>12</sub> influence nucleic acid synthesis through participation in the biosynthesis of purine and pyrimidine bases<sup>3</sup> the protection by these vitamins against antibiotic action in micro-organisms might seem explicable on this basis.

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#### Discussion

DR. P. S. SARMA: Could the use of antibiotic and antivitamin B<sub>12</sub> throw more light on the role of tetracycline antibiotics in nucleotide metabolism of micro-organisms?

DR. A. SREENIVASAN: The effect of antivitamins has already been studied and reported<sup>3</sup>. A combination of antibiotic and antivitamin may not be expected to give information in addition to their use individually.



# Terramycin and Growth : Part I—A Study of the Relationship of Pteroyl Glutamic Acid and Vitamin B<sub>12</sub> to Growth Stimulation in Weanling Rat by Dietary Terramycin

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The relationship between folic acid, vitamin B<sub>12</sub> and terramycin in weanling rats reared on a low methionine-low choline diet has been studied.

Dietary terramycin stimulates growth of young rats reared on low methionine-low choline diet. Vitamin B<sub>12</sub> and folic acid exert a comparable effect and enhance the effect of the antibiotic further.

Consequent on the improved growth there is an increase in the liver levels of total choline, methionine, vitamin B<sub>12</sub> and folic acid. Circulatory methionine in plasma was more in the basal unsupplemented group. The observations bring out the effect of terramycin on the metabolism of methionine and choline presumably through increased availability of folic acid and vitamin B<sub>12</sub>.

Following the observation of Stockstad *et al.*<sup>1</sup> several workers have demonstrated that the addition of minimal amounts of certain antibiotics to an otherwise adequate diet will significantly increase the growth rate of a variety of animals. In a number of studies, it was emphasized that the type of basal diet employed could have an effect on the ability of antibiotics to stimulate growth. Thus both aureomycin and terramycin stimulate growth in rats only under certain dietary inadequacies<sup>2</sup>. In particular, they give a response with diets providing limiting amounts of the B vitamins<sup>3-7</sup>; their effect is also related to protein metabolism<sup>8</sup> and to protein source<sup>9</sup>. Simultaneous supplementation of certain diets with vitamin B<sub>12</sub> and aureomycin resulted in greater growth and higher food efficiency than did the addition of the antibiotic alone<sup>10</sup>. According to Sauberlich<sup>11</sup>, the growth-promoting effect of antibiotics in rats is related to the amino acid composition of the diet. Pecora<sup>12</sup> reported that protein efficiency ratios were improved in rats receiving a rice-lysine diet and an antibiotic. Baxter and Campbell<sup>13</sup> observed that renal lesions and mortality in rats caused by a purified diet deficient in choline were largely prevented by supplementation of the diet with rather high levels of aureomycin. It has been shown that the growth stimulatory effect of antibiotics is due to increased food intake, the effect not being observed

TABLE 1—TERRAMYCIN AND GROWTH

GROUP NO.	SUPPLEMENT/KG. OF BASAL DIET	MEAN INCREASE IN WEIGHT (G.) AT END OF	
		4 weeks	8 weeks
I	Nil	37.0 ± 2.4	74.2 ± 3.1
II	Terramycin (20 mg.)	53.2 ± 3.3	108.8 ± 5.3
III	Terramycin (50 mg.)	59.3 ± 4.5	104.3 ± 5.6
IV	PGA (1 mg.) + Vitamin B <sub>12</sub> (200 µg.)	56.0 ± 4.6	98.0 ± 8.1
V	Terramycin (20 mg.) + PGA (1 mg.) + Vitamin B <sub>12</sub> (200 µg.)	70.8 ± 5.7	116.4 ± 4.3
VI	Terramycin (50 mg.) + PGA (1 mg.) + Vitamin B <sub>12</sub> (200 µg.)	61.5 ± 2.5	126.8 ± 8.3

when animals were pair-fed to those not receiving the drug<sup>14-15</sup>. There is also evidence to suggest that antibiotics may act by (i) suppressing the growth of certain intestinal micro-organisms which deprive the host of essential nutrients<sup>16</sup>; (ii) encouraging the growth of other organisms which biosynthesize some of the essential food factors<sup>17</sup>; or (iii) altering the absorption characteristics of the intestinal wall favourably for the host animal<sup>18</sup>. Whatever may be the mechanisms of antibiotics action it would seem that in various species they exert a sparing action on or cause increased availability of certain vitamins, especially folic acid (PGA) and vitamin B<sub>12</sub> and some essential amino acids whose metabolism is in turn influenced by these vitamins.

The present experiments were designed to bring out the relationship between PGA, vitamin B<sub>12</sub> and terramycin in weanling rats reared on a low methionine-low choline diet. This diet was based on Bengal gram (*Cicer arietinum*), known to be deficient in methionine<sup>19</sup> and was fortified with choline at a very low level.

### EXPERIMENTAL AND RESULTS

The composition of the basal diet (percentages) was as follows: sifted gram flour, 90; sesame oil, 5; U.S.P. salt mixture, 4 and vitaminized sucrose\*, 1. Choline chloride was separately incorporated in the basal diet at a level of 100 mg./kg. diet.

The basal diet on analysis was found to contain 18.6 per cent protein and 0.5 per cent methionine and cystine<sup>21</sup>. Vitamin A was added to the diet at a level of 5,000 I.U./kg.

Male albino rats (Haffkine strain), about 50 g. in weight, were grouped, six to a group, and fed *ad libitum* the basal diet with or without the supplements.

\* The vitaminized sucrose was the same as that of Artom and Cornatzer<sup>20</sup> with the omission of PGA and vitamin B<sub>12</sub> and contained per gram, thiamine hydrochloride, 0.4 mg.; riboflavin, 0.8 mg.; niacin, 5.0 mg.; calcium pantothenate, 2.5 mg.; *p*-aminobenzoic acid, 2.5 mg.; inositol 10.0 mg.; menadione 0.5 mg.; biotin, 0.05 mg. and pyridoxin, 0.4 mg.

The animals were weighed twice weekly and the average increases in weight at the end of 4 and 8 weeks respectively on the different diets were recorded (Table 1).

At end of the 8 week period, the rats were anaesthetized with approximately 10 mg./kg. body wt. phenobarbitone in ethanol, administered intraperitoneally in one single dose and, after sacrifice, the blood was drawn from the portal vein and immediately poured into 1 ml. of 3.5 per cent sodium citrate solution. The animals were then bled thoroughly and the liver removed, dried between folds of filter paper to remove blood clots and immediately weighed.

Protein-free blood filtrates<sup>22</sup> and liver filtrates<sup>23</sup> were prepared and free methionine was estimated microbiologically in the neutralized tungstic acid filtrates by a micro-method<sup>24</sup> using a Cannon-Dispenser-Titrator assembly. From 0.04 to 0.2 ml. of samples were dispensed into a series of tubes followed by requisite quantities of water to make 0.2 ml. and 0.2 ml. of double strength assay medium and values for methionine were obtained from a reference standard of dl-methionine.

Samples for the assay of PGA in blood and liver were prepared after autolysis in phosphate buffer at pH 7.4 under toluene for 20 hr.<sup>25,26</sup> and estimated using *Streptococcus fecalis* R and the improved assay medium of Mitbander and Sreenivasan<sup>27</sup>.

Samples for the assay of vitamin B<sub>12</sub> were prepared after liberation in blood by papain<sup>28</sup> and in liver by heat coagulation<sup>29</sup>. Vitamin B<sub>12</sub> was estimated using *Escherichia coli* B<sub>12</sub> mutant<sup>30</sup>.

The procedure adopted for the liberation of choline was a modification of an earlier method of Lueke and Pearson<sup>31</sup>. To 5 ml. of liver homogenate corresponding to 1 g. fresh wt. liver 5 ml. of 3 per cent sulphuric acid was added and the mixture autoclaved at 15-lb. pressure for 2 hr. The hydrolysate was neutralized with barium hydroxide to pH 5.0-5.5, made to a convenient volume and filtered. Choline was estimated after adsorption on activated Decalso and elution by 5 per cent sodium chloride according to the procedure of Appelton *et al.*<sup>32</sup>

The results obtained with liver samples are recorded in Table 2 and those with blood samples in Table 3.

## DISCUSSION

The average growth rate on the basal diet is low, but this is enhanced when the diet is supplemented with terramycin at both levels. Since the basal diet is limiting for growth only with respect to PGA and B<sub>12</sub> among the vitamins, it would seem that the antibiotic spares the requirement for these vitamins. Fortification of the basal diet with a mixture of both PGA and B<sub>12</sub> results in an effect comparable to that with the antibiotic. However, in the groups fed with the antibiotic and the two vitamins, there is additional growth effect which is seen better with low level of antibiotic supplementation (Gr. 5) than with the higher level (Gr. 6). The growth trend is the same during the first and second 4-week periods.



TABLE 2—LIVER LEVELS OF CHOLINE, METHIONINE, PGA AND VITAMIN B<sub>12</sub>

GROUP NO.	SUPPLEMENTS/KG. OF BASAL DIET	TOTAL CHOLINE mg./g. <i>fresh wt.</i>	FREE METHIONINE μg./g. <i>fresh wt.</i>	TOTAL PGA μg./g. <i>dry wt.</i>	VITAMIN B <sub>12</sub> μg./g. <i>dry wt.</i>
1	Nil	4.32 ± 0.14	62.3 ± 0.12	3.94 ± 0.12	280 ± 20.2
2	Terramycin (20 mg.)	4.64 ± 0.07	64.7 ± 0.20	4.24 ± 0.07	340 ± 34.5
3	Terramycin (50 mg.)	4.93 ± 0.18	63.4 ± 0.31	4.72 ± 0.18	380 ± 21.6
4	PGA (1 mg.) + vitamin B <sub>12</sub> (200 μg.)	5.40 ± 0.23	65.2 ± 0.18	5.01 ± 0.26	400 ± 12.2
5	Terramycin (20 mg.) + PGA (1 mg.) + vitamin B <sub>12</sub> (200 μg.)	5.54 ± 0.17	65.6 ± 0.04	5.80 ± 0.04	400 ± 30.6
6	Terramycin (50 mg.) + PGA (1 mg.) + vitamin B <sub>12</sub> (200 μg.)	5.67 ± 0.09	66.5 ± 0.08	6.80 ± 0.24	470 ± 10.0

TABLE 3—BLOOD LEVELS OF METHIONINE, PGA AND VITAMIN B<sub>12</sub>

GROUP NO.	SUPPLEMENTS/KG. OF BASAL DIET	FREE METHIONINE μg./ml.	TOTAL PGA μg./ml.	VITAMIN B <sub>12</sub> mμg./ml.
1	Nil	36.3 ± 0.10	8.25 ± 0.14	225 ± 8.0
2	Terramycin (20 mg.)	32.8 ± 0.16	9.40 ± 0.2	253 ± 12.2
3	Terramycin (50 mg.)	29.5 ± 0.22	9.80 ± 0.08	268 ± 16.3
4	PGA (1 mg.) + B <sub>12</sub> (200 μg.)	27.4 ± 0.14	10.2 ± 0.7	297 ± 30.2
5	Terramycin (20 mg.) + PGA (1 mg.) + B <sub>12</sub> (200 μg.)	24.3 ± 0.24	10.5 ± 0.26	315 ± 10.2
6	Terramycin (50 mg.) + PGA (1 mg.) + B <sub>12</sub> (200 μg.)	21.7 ± 0.12	12.2 ± 0.18	320 ± 20.4

Dietary terramycin as well as supplements of PGA and B<sub>12</sub> result in increased liver phospholipids as shown from the values for total choline in the liver. Together, their effects are more. These observations bring out the well-known significance of the vitamins in methyl metabolism. The influence of PGA on phospholipids has been shown directly by Fatterpaker *et al.*<sup>33</sup> The observations thus afford presumptive proof of the effect of terramycin on growth as being due to a control in the economy of these metabolites.

The free methionine increases in liver and decreases in plasma with the addition of antibiotic and of PGA and B<sub>12</sub> singly or together. Charkey *et al.*<sup>34</sup> have reported that vitamin B<sub>12</sub> functions in the mobilization of circulatory amino acids.

The higher values for PGA and B<sub>12</sub> in both plasma and liver of the groups supplemented with these vitamins are to be expected, since the basal diet was devoid of these vitamin additions. The similar enhanced values in the antibiotic supplemented groups would point to a control by the drug of the intestinal microflora in the direction of improved availability of these vitamins. It may be inferred, therefore that the beneficial effect of terramycin with respect to the two vitamins on the low-methionine low-choline diet devoid of PGA and B<sub>12</sub> is due to an increased availability to the host animal of intestinally synthesized PGA and vitamin B<sub>12</sub> rather than to increased absorption of the vitamins alone. The data on choline and methionine are explicable on the basis of their interdependence with the metabolism of PGA and B<sub>12</sub>. The fact that the stimulatory effect of terramycin is enhanced by supplement of PGA and vitamin B<sub>12</sub> would however point to a function for the antibiotic in other directions as well. It has been observed, for example, that in the groups fed by the antibiotic there is a relatively greater conservation of liver protein during protein fasting of the animals (unpublished work). Studies, in progress, on the effect of antibiotic feeding on liver xanthine oxidase activity and liver levels of flavin adenine dinucleotide (FAD) and coenzyme A (CoA) and on mitochondrial coupling of oxidation to biosynthetic activities also point to additional functions.

In these studies, the effect of PGA and B<sub>12</sub> *vis-a-vis* the effect of terramycin on growth of young rats was observed together in view of their known interrelationships in various metabolic processes<sup>35</sup>. The studies are being extended to ascertain the relative contribution of the two vitamins on antibiotic stimulation to growth.

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# Terramycin and Growth: Part II—The Effect of Terramycin on the Absorption of Vitamin B<sub>12</sub> by *Escherichia coli*

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The influence of terramycin on the uptake of vitamin B<sub>12</sub> by *E. coli* has been discussed. Terramycin considerably inhibits the uptake of vitamin B<sub>12</sub> by *E. coli*.

It has been suggested that antibiotics stimulate growth of certain animals by suppressing those components of the intestinal microflora which normally deprive the host of significant quantities of ingested nutrients<sup>1-3</sup>. It has also been claimed that antibiotics, by suppressing some members of the gastrointestinal population, may permit the more extensive proliferation of organisms which, by their biosynthetic activities, contribute materially to the nutrition of the host. Thus, Elam *et al.*<sup>4</sup> found a decrease in total number of *Clostridia* per gram of feces along with growth stimulation on antibiotic feeding. Certain colon organisms are known to be avid consumers of vitamin B<sub>12</sub> which is not necessarily utilized in their metabolism<sup>5,6</sup>. In an earlier paper<sup>7</sup> it was observed that dietary terramycin spares the requirement of vitamin B<sub>12</sub> and folic acid (PGA) for rats reared on a low methionine-low choline diet. A study of vitamin B<sub>12</sub> absorption by a strain of *Escherichia coli* Mcleod, a fecal organism, was undertaken in an attempt to explain the vitamin B<sub>12</sub> sparing action of terramycin.

## EXPERIMENTAL

Cells of *E. coli* Mcleod were grown in salts-synthetic medium of Green and Sevag<sup>8</sup> and, after 20 hr. growth, were harvested by centrifugation, washed once with distilled water and made into a suspension (20 mg. dry wt. cells/ml.). The reaction system consisted of 1 ml. of vitamin B<sub>12</sub> solution containing 1,000 mμg. vitamin B<sub>12</sub>, 3 ml. of M/20 phosphate buffer pH 7.4 1 ml. of cell suspension and 1 ml. of distilled water or terramycin solution (10 or 100 μg. as stated). After incubation at 37°C. for 2 hr., the cells were removed by centrifugation and washed once with distilled water. The supernatant and washings were combined, steamed for 10 min. and made up to volume. The residual B<sub>12</sub> was assayed using *E. coli* B<sub>12</sub> mutant.

TABLE 1—INFLUENCE OF TERRAMYCIN ON VITAMIN B<sub>12</sub> UPTAKE BY *E. COLI*

TERRAMYCIN PER 6 ML. REACTION SYSTEM $\mu\text{g.}$	B <sub>12</sub> UPTAKE BY 20 MG. DRY WT. CELLS IN 3 HR. $\text{m}\mu\text{g.}$	INHIBITION %
None	520	..
10	504	3.0
100	325	37.5

In three separate experiments, the values for B<sub>12</sub> uptake in 2 hr. by cells (equivalent to 20 mg. dry wt.) were 346, 352 and 380  $\text{m}\mu\text{g.}$  respectively working to 36 per cent absorption. Increasing the time of incubation to 3 hr. resulted in an average absorption of 52 per cent.

Several experiments were carried out to ascertain the effect of terramycin on this absorption. It was observed that the organism was sensitive to the antibiotic in low concentration, the minimum inhibitory concentration as determined by serial dilution method in salts-synthetic medium being 0.52  $\mu\text{g./ml.}$  The assay organism *E. coli* B<sub>12</sub> mutant was also inhibited in its growth by terramycin. The effect of terramycin was therefore studied by estimation of absorbed vitamin B<sub>12</sub> after liberation from cells of *E. coli* by papain digestion<sup>10</sup> as follows.

The washed cells were suspended in 4 ml. distilled water ; 6 ml. of 0.1 M acetate buffer pH 4.5 was added, followed by 1 ml. of a dispersion of 30 mg. papain (Nutrition Biochemicals) in acetate buffer. The digestion was carried out for 18 hr. at 37° with toluene addition. The cells were then steamed for 10 min., made to volume and centrifuged. Vitamin B<sub>12</sub> was estimated in the centrifugates by *Lactobacillus leichmannii*<sup>11</sup>. The results obtained are recorded below and represent average values of three independent experiments.

## DISCUSSION

The fairly low minimum inhibitory concentration of terramycin against *E. coli* suggests the possibility of its partial elimination in the intestinal tract as in the case of streptomycin<sup>12</sup> and aureomycin<sup>13</sup>. Increased amounts of vitamin B<sub>12</sub> may thus be made available to the host animal on antibiotic feeding. The interference by terramycin with the absorption of vitamin B<sub>12</sub> by *E. coli* *in vitro* would also suggest that more of the vitamin is available for absorption by the animal in presence of the antibiotic. The higher values for vitamin B<sub>12</sub> in blood and liver obtained with antibiotic feeding<sup>7</sup> support this possibility. Whether similar effects are observable with other nutrients and fecal organisms is being ascertained.

## SUMMARY

Resting cells of *E. coli* Mcleod take up considerable amounts of vitamin B<sub>12</sub> from the surrounding medium. This uptake is interfered with by terra-

mycin when present in the medium in small concentrations not completely inhibitory to the organism.

#### ACKNOWLEDGMENT

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TABLE 3—EFFECT OF AUREOMYCIN AND/OR VITAMIN B<sub>12</sub> ON BIOLOGICAL VALUE AND DIGESTIBILITY COEFFICIENT OF PROTEIN OF POOR RICE DIET\*  
(No. of rats examined, 6)

DIET	FIGURES REPRESENT AVERAGES / 4-DAY PERIOD / RAT			BIOLOGICAL VALUE mg.	DIGESTIBILITY COEFFICIENT
	Average No. intake mg.	Average urinary N mg.	Average faecal N mg.		
Basal diet (B.D.)	594.8	171.6 (18.2)	181.0 (30.3)	65.51 ± 0.56	73.92 ± 0.94
B.D. + aureomycin	631.5	120.8 (8.9)	158.7 (15.9)	77.13 ± 1.50	77.42 ± 1.22
B.D. + vitamin B <sub>12</sub>	655.2	124.3 (7.9)	153.6 (16.5)	76.98 ± 0.89	78.8 ± 1.15
B.D. + aureomycin + vitamin B <sub>12</sub>	667.4	109.5 (9.1)	129.2 (17.4)	80.89 ± 0.89	83.26 ± 1.40

\* Values in parentheses are for N-free diet

and were fed the diets as indicated in Table 2. The animals were weighed twice a week and the tests were continued for 9 weeks.

Nitrogen balance determinations were made on male rats, weighing about 130 g., by the method of Mitchell<sup>7</sup> as adopted by Swaminathan<sup>8</sup>. Faecal and urinary nitrogen estimations were carried out on a N-free diet with and without aureomycin and/or vitamin B<sub>12</sub>. The animals were transferred to stock diet for a rest period of 7 days. This was followed by 7 days feeding of test diets. Faecal and urinary collections of last four days were considered for biological value and digestibility coefficient calculations. The supplements of vitamin B-complex added to the diets in this series of tests provided the following quantities, mg. per kg. of diet: thiamine HCl, 1.75; riboflavin, 2.8; niacin, 14; pyridoxine HCl, 1.75; calcium pantothenate, 14; folic acid, 0.7; inositol, 35 and *p*-aminobenzoic acid, 35.

### RESULTS AND DISCUSSION

The results of growth response to aureomycin and/or vitamin B<sub>12</sub>, compared to controls, over a period of 9 weeks are summarized in Table 2.

Aureomycin produced a positive growth stimulation. Vitamin B<sub>12</sub> did not improve the growth when fed with poor rice diet, since the weight increase throughout the experimental period was almost the same as in the control group. Similar findings were reported earlier by Pecora<sup>6</sup>. However, the latter worker has shown that vitamin B<sub>12</sub> enhances the growth effect of aureomycin when supplemented with it. This favourable influence of vitamin B<sub>12</sub> has not been observed (Table 2, Group 4). Both the tests (Groups 3 & 4) indicate that vitamin B<sub>12</sub> has no growth property in poor rice diets. In the early period of the experiment, some variation in the food consumption by animals in all groups was noted. Probably due to this, no significant difference in the growth rates at the three weeks period is observed between the test diets and the control diet.

Nitrogen balance studies are based on a period of four days collections of urine and faeces as given in Table 3. Endogenous nitrogen metabolism values for urine and faeces on N-free diet with and without supplement are shown in parentheses with corresponding values for test diets. Although the animals of all the groups in nitrogen balance studies were given diets and water *ad libitum*, the average nitrogen intake of the test groups is significantly higher than the control group (Table 3). Amongst test groups, the nitrogen intake was very nearly the same. Aureomycin and vitamin B<sub>12</sub> increased, almost to the same degree the biological value and the digestibility coefficient by improving the nitrogen assimilation of rice diets. When rice diet was supplemented with both the aureomycin and vitamin B<sub>12</sub> they further enhanced the biological value and digestibility coefficient to 81 and 83 respectively. However, such effect was not observed on growth (Table 2). Further, it will be seen that aureomycin and vitamin B<sub>12</sub> considerably reduced the excretion of nitrogen on N-free diet, indicating that these substances favourably effect endogenous nitrogen metabolism.

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# Effect of Antibiotics on Vitamin A Metabolism, Growth, Carcass Composition and Haematological Values in Rats

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The effect of antibiotics (penicillin, terramycin, streptomycin and aureomycin) on sparing of vitamin A and on the conversion of  $\beta$ -carotene to vitamin A was studied. The comparative growth promoting property of these substances, in the presence and absence of vitamin A, was also investigated. In addition, their effects on carcass composition, protein and fat deposition in liver and haematological values were observed.

The antibiotics did not influence the vitamin A depletion period in weanling rats. The conversion of  $\beta$ -carotene to vitamin A occurs unaltered in animals previously fed with antibiotics. Increased growth rates of rats receiving antibiotics supplement were obtained in the following descending order : aureomycin, penicillin/terramycin and streptomycin. Carcass analyses showed increase in the protein and fat contents of the antibiotic fed groups.

It has been reported that antibiotics influence favourably the utilization of fat soluble vitamins in animals. The first observation in this connection was made by Burgess *et al.*<sup>1</sup>, who found an increase in liver vitamin A and serum carotenoids of chicks receiving dietary penicillin. Similar findings were reported by Coates<sup>2</sup> but she noted that chicks which did not show growth response to the antibiotic, also did not show higher stores of vitamin A in livers compared to the control groups. Barber *et al.*<sup>3</sup> showed that store of vitamin A in the livers of pigs given aureomycin is the highest but could not obtain similar results with penicillin and streptomycin. Subsequently, Hartsook *et al.*<sup>4</sup> demonstrated that aureomycin had no sparing action on vitamin A in growing male rat.

The conversion of carotenoid pigments to vitamin A occurs in the intestinal wall of rat<sup>5-7</sup> and chick<sup>8</sup>. In her preliminary experiments on infected chicks, Coates<sup>2</sup> found that birds treated with penicillin converted  $\beta$ -carotene to vitamin A in the intestine more efficiently than the untreated controls. Since antibiotics are known to alter the intestinal microflora, it is probable that a different mechanism of absorption and transformation to vitamin A may occur in antibiotic treated animals.

In view of the above contradictory reports, it was thought interesting to examine in detail the effects of four common antibiotics viz., penicillin, streptomycin, aureomycin and terramycin on vitamin A and  $\beta$ -carotene metabolism



in rat. In addition, results on growth, carcass composition and haematological values are also included in this paper.

### EXPERIMENTAL

In these investigations 330 Haffkine Inbred male albino rats weighing about 35–40 g. were used. The animals were maintained on a vitamin A free synthetic diet of Bliss and Gyorgy<sup>9</sup> except that casein was included at 10 per cent level. Vitamin supplements were given separately. In experimental diets, the antibiotic was incorporated at the level of 25 mg./kg. of basal diet by gradual dilution technique. Such diets were prepared in small amounts so as to last for 3 or 4 days and stored in a refrigerator to prevent any deterioration in the antibiotic. In all experiments, the animals were housed in individual cages and were fed diet *ad libitum*.

To determine the vitamin A sparing action of penicillin, terramycin, streptomycin and aureomycin, two independent procedures were followed. In one, the period taken to produce a typical syndrome of avitaminosis A, characterized by loss in weight and/or xerophthalmia, in rats receiving vitamin A deficient diets with and without antibiotics was noted. For this purpose, 30 rats were divided into 5 groups. One group served as a control, while the other four groups received diet containing one of the four antibiotics. The animals were weighed twice a week and killed at the end of the experiment and their liver weights were noted. In another series, similar effect was studied by assaying the livers for vitamin A stores of antibiotic treated and untreated vitamin A-depleted animals which were given vitamin A orally at four levels: 150, 250, 500 and 1,000 I.U./rat/week in three doses. Thus, 20 groups of 6 animals each including the controls were used for this study. The animals receiving 150 I.U. vitamin A/week were killed at the end of 8 weeks, while the animals fed vitamin A at the other three levels were sacrificed at the end of four weeks. Livers were removed, cleaned of adhering tissues and blood and weighed. The whole liver was then saponified by boiling under reflux for half an hour with 20 ml. of 95 per cent alcohol and 1 ml. of 60 per cent caustic potash per gram of liver. The unsaponifiable material was extracted with petroleum ether. The latter was removed under vacuum and the residue dissolved in known volume of chloroform. Vitamin A was determined in 0.2 ml. aliquot of chloroform solution by antimony trichloride reaction.

For testing the effect of antibiotics on the metabolism of carotene to vitamin A, 150 rats were divided into 5 lots of 30 each. One lot was put on vitamin A-free diet alone while remaining four received vitamin A-free diet with one of the four antibiotics, when vitamin A deficiency was produced, they were dosed with 250  $\mu$ g. of carotene in 0.5 ml. of groundnut oil, containing 1 mg. tocopherols per ml. by stomach tube. The animals were killed at the intervals of 4, 6, 8, 12 and 24 hr. after dosing and the livers and intestines were removed. The latter were washed out with about 60 ml. of normal saline using a blunt needle syringe. The tissues and intestinal washings were analysed for vitamin A as before. Carotene was determined wherever necessary.

TABLE 1—EFFECT OF ANTIBIOTICS ON BODY AND LIVER WEIGHTS, AND ON DEPLETION TIME OF RATS FED VITAMIN A-FREE DIET

DIET	NO. OF RATS	INITIAL WT. g.	FINAL WT. g.	GAIN IN WT. g.	LIVER WT. g.	DEPLETION PERIOD days
Vitamin A-free	6	30.4	74.6	42.2	3.15	41.8
Vitamin A-free + penicillin	6	30.8	75.2	44.4	3.077	41.5
Vitamin A-free + terramycin	6	30.3	74.7	44.4	3.092	44.8
Vitamin A-free + streptomycin	6	30.3	73.9	43.6	3.119	41.5
Vitamin A-free + aureomycin	6	30.4	75.1	44.7	3.096	41.5

TABLE 2—EFFECT OF ANTIBIOTICS ON LIVER VITAMIN A STORAGE OF RATS ADMINISTERED VITAMIN A

DIET	DOSE/WEEK I.U.	NO. OF RATS	PERIOD weeks	LIVER WT. g.	TOTAL LIVER VITAMIN A I.U.	VITAMIN A/g. OF FRESH LIVER I.U.
Vitamin A-free	150	6	8	4.523	65.8	14.6
	250	6	4	3.429	109.3	31.9
	500	6	4	3.301	236.0	71.5
	1,000	6	4	3.352	881.8	267.2
Vitamin A-free + penicillin	150	6	8	5.414	69.7	12.9
	250	6	4	3.692	115.0	31.2
	500	6	4	3.67	259.9	70.7
	1,000	6	4	3.735	973.0	264.1
Vitamin A-free + terramycin	150	6	8	5.366	70.3	13.2
	250	6	4	3.468	113.0	32.5
	500	6	4	3.63	274.7	74.6
	1,000	6	4	3.431	951.5	280.9
Vitamin A-free + streptomycin	150	6	8	5.23	69.7	13.3
	250	6	4	3.433	111.2	32.4
	500	6	4	3.575	260.4	72.6
	1,000	6	4	3.318	870.2	267.1
Vitamin A-free + aureomycin	150	6	8	5.727	73.5	12.8
	250	6	4	3.96	118.2	30.3
	500	6	4	4.076	284.0	70.4
	1,000	6	4	4.14	1,079.8	263.7

In another series of experiments designed to study the growth effect, the control group did not receive any supplement of antibiotic, while each test group received one different antibiotic along with the diet. Animals in all the groups were given 150 I.U. vitamin A per week in three doses. The animals were weighed as before twice a week, and records of food consumption were also maintained. At the end of 8 weeks, the animals were anesthetized, and blood drawn from the heart with a capillary pipette for haematological values and proteins. Liver also was removed for protein and fat estimations.

TABLE 3—EFFECT OF ANTIBIOTICS ON CONVERSION OF  $\beta$ -CAROTENE TO VITAMIN A IN VITAMIN A DEPLETED RATS AFTER ADMINISTERING 250  $\mu$ g. CAROTENE IN GROUNDNUT OIL

(Number of rats examined, 6)

PERIOD (hr.)	INTESTINAL TRACT CAROTENE		INTESTINAL WASHINGS		INTESTINAL TRACT VITAMIN A		LIVER VITAMIN A	
	per rat mcg.	per g. fresh tissue mcg.	Carotene/ rat mcg.	Vitamin A/rat I.U.	per rat I.U.	per g. fresh tissue I.U.	per rat I.U.	per g. fresh tissue I.U.
Vitamin A-free diet—no antibiotic								
4	18.2	3.6	31.9	N.D.	18.2	3.5	10.5	3.2
6	32.8	6.3	59.4	N.D.	35.7	6.7	19.2	4.5
8	38.1	7.3	43.5	N.D.	30.4	5.3	33.6	8.8
12	19.9	4.3	25.6	N.D.	21.4	4.1	43.8	10.6
24	5.3	0.9	5.0	N.D.	11.2	2.1	73.0	17.1
Vitamin A-free diet plus penicillin								
4	17.3	3.8	31.2	N.D.	18.7	4.1	11.4	3.5
6	32.3	6.1	63.0	N.D.	34.7	6.6	19.1	4.5
8	35.9	6.9	49.6	N.D.	30.6	5.2	36.4	9.8
12	20.9	4.4	23.5	N.D.	21.2	4.1	40.2	10.2
24	4.8	0.7	5.1	N.D.	11.6	2.2	74.9	16.9
Vitamin A-free diet plus terramycin								
4	18.5	4.0	28.2	N.D.	20.6	4.4	11.2	3.3
6	33.1	6.0	65.2	N.D.	32.6	6.2	19.9	4.4
8	33.2	6.4	48.7	N.D.	32.7	5.3	36.3	9.6
12	20.8	4.2	23.4	N.D.	21.8	4.4	42.3	10.3
24	5.5	0.8	4.5	N.D.	11.7	2.2	71.1	16.7
Vitamin A-free diet plus streptomycin								
4	19.1	4.0	26.5	N.D.	20.6	4.1	11.3	3.4
6	31.7	6.0	67.6	N.D.	31.7	6.2	19.8	4.5
8	33.1	6.5	49.2	N.D.	32.8	5.3	36.5	9.8
12	21.36	4.6	22.3	N.D.	21.4	4.3	39.1	9.7
24	5.0	0.8	5.3	N.D.	10.6	2.1	72.3	16.0
Vitamin A-free diet plus aureomycin								
4	19.9	3.9	29.5	N.D.	18.5	3.6	10.8	3.5
6	32.8	5.9	67.8	N.D.	31.9	6.1	19.8	4.3
8	32.7	6.5	48.3	N.D.	32.5	5.0	35.8	9.7
12	20.5	4.2	21.5	N.D.	21.0	4.0	39.5	10.0
24	5.6	0.9	4.9	N.D.	11.7	2.2	75.0	11.9

N.D.—Amount not detectable



The alimentary canal was washed off the undigested food and faeces. The carcass was then minced in a mincing machine until homogeneous and, nitrogen, fat, and ash were determined in duplicate on an aliquot.

## RESULTS

### Effect of antibiotics on vitamin A depletion period and on vitamin A liver storage

The effect of penicillin, terramycin, streptomycin and aureomycin on the utilization of vitamin A was studied by two procedures as mentioned earlier. The results of the first method are given in Table 1.

It will be noted from Table 1 that antibiotics do not seem to exert any growth promoting property in the absence of vitamin A, since the gain in weight in control as well as test groups was practically the same. The same holds good with regard to liver weights. The symptoms of vitamin A deficiency appeared on an average after 41 days in all groups except the terramycin group. In the latter group, it took about 44 days. However, this difference is not statistically significant. Thus, it is observed that antibiotics do not modify the utilization of vitamin A.

The data on the foregoing effect of antibiotics obtained by following another procedure are shown in Table 2.

When 150 or 250 I.U. vitamin A/week was administered, practically no difference was observed in the liver storage of vitamin A in control and experimental groups. At higher levels of vitamin A dosing (500 and 1,000 I.U./week), there appeared more vitamin A in the livers of antibiotic fed animals than the control. However, this difference is not borne out when the figures are evaluated I.U./g. of the fresh liver tissue.

### Influence of antibiotics on conversion of $\beta$ -carotene to vitamin A

Although the exact mechanism of action of antibiotics in promoting growth of animals is not understood, the consensus of opinion is that they act by altering the microflora of the intestinal gut. In order to determine, whether the normal bacterial flora play a role in the transformation of carotene to vitamin A, experiments were carried out on vitamin A-depleted rats to whom antibiotics had been given along with the depletion diets, before the tests were made. These experiments are summarized in Table 3.

In all the groups, more vitamin A appeared in the intestinal wall than in the liver, 4 to 8 hr. after the administration of carotene. In the subsequent periods, the amount of vitamin A in the liver was found to exceed that in the intestinal wall. No detectable amount of vitamin A was found in the washings of the intestinal tract. These observations indicate that the transformation of carotene to vitamin A still proceeds normally, when the usual bacterial flora are changed in the gastro-intestinal tract by feeding antibiotics.

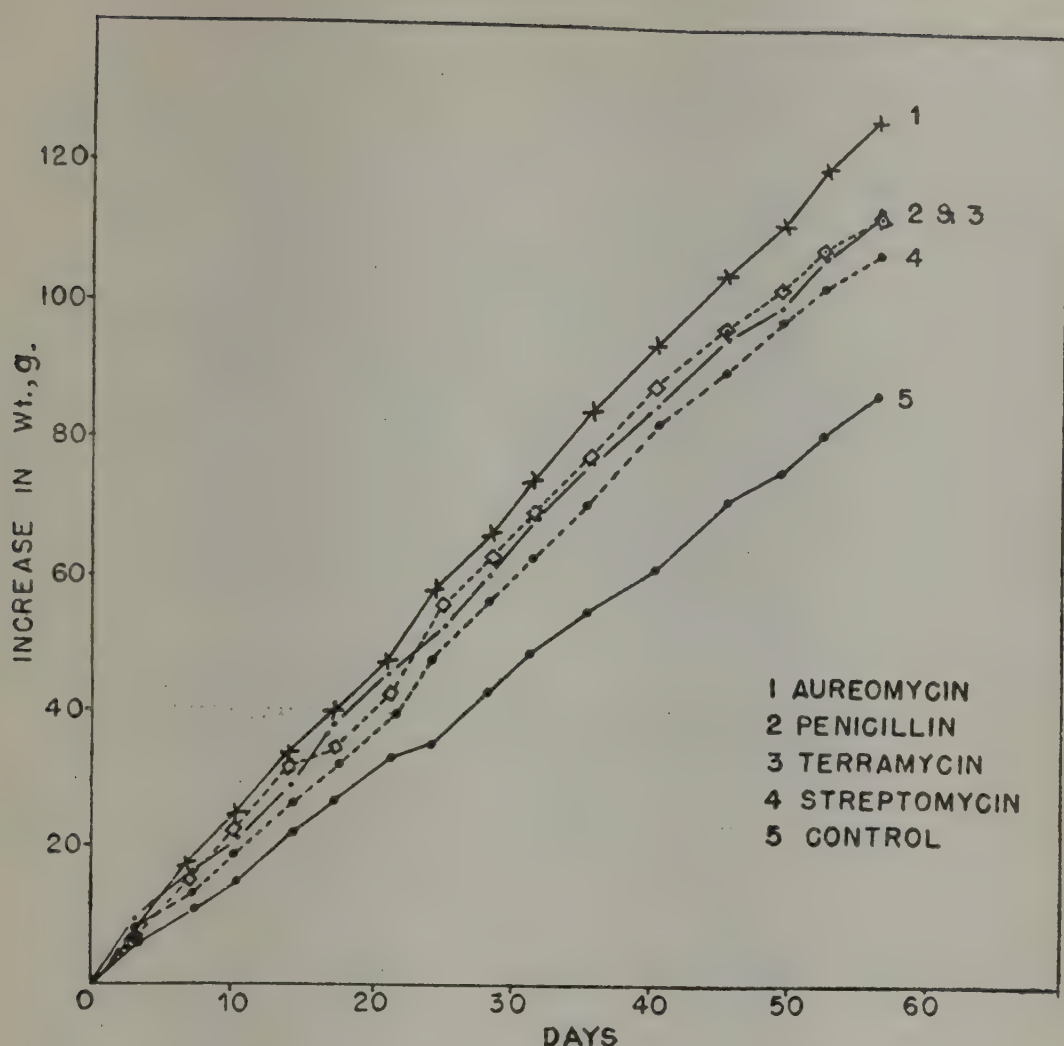


FIG. 1—GROWTH RESPONSE OF RATS FED ON DIFFERENT ANTIBIOTICS (25 MG./KG. DIET)

### Influence of antibiotics on body weight, carcass composition and haematological values

The rats receiving antibiotic supplements had significantly better growth throughout the experiment than the rats receiving basal diet (Fig. 1). Amongst antibiotics fed groups, gain in weight was maximum in aureomycin group. Penicillin and terramycin showed almost the same effect; while streptomycin was last in the order.

The carcass analyses showed an increase in protein and fat values of experimental animals. Similar increase in liver protein and fat is also noticed. However, no difference was observed in the ash content of various groups including control.

In this series of experiments, the effect of antibiotics on blood constituents was also studied. Proteins and haemoglobin showed some increase in the experimental groups. But this increase was not significant. Similarly, no effect on R.B.C. and W.B.C. counts was noted. These observations are included in Table 4.

## DISCUSSION

Two series of experiments were carried out to test the efficacy of four antibiotics on vitamin A utilization in rat. The results of both the tests indicate that the antibiotics have no vitamin A sparing action. While studying their effect on vitamin A-depletion period, it was noted that it took about 41 days in all groups, except the terramycin group which took about 44 days. It is the experience in this laboratory that Haffkine Inbred weanling rats usually take 38 to 42 days to produce vitamin A deficiency characterized either by continuous loss in weight and/or xerophthalmia, when fed vitamin A-free diet. If the antibiotics had any sparing action on vitamin A utilization, there would have been delayed production of vitamin A deficiency in experimental animals compared to the control group. On the other hand, Hartsook *et al.*<sup>4</sup> have reported an enhanced production of typical syndrome of avitaminosis A in rats fed aureomycin. Similarly, Burgess *et al.*<sup>1</sup> have indicated earlier production of vitamin A deficiency in chicks on vitamin A-free diet plus antibiotic than on vitamin A-deficient diet alone.

TABLE 4—EFFECT OF ANTIBIOTICS ON BODY WEIGHT, CARCASS COMPOSITION, LIVER PROTEINS & FATS AND HAEMATOLOGICAL VALUES

DIET	BASAL DIET (B.D.)	B.D. + PENICILLIN	B.D. + TERRA- MYCIN	B.D. + STREPTO- MYCIN	B.D. + AUREO- MYCIN
Rat (No.)	6	6	6	6	6
Duration (weeks)	8	8	8	8	8
Final body wt. (g.)	120.1	145.3	144.1	139.2	159
Wt. gain (g.)	87.1	113.6	113.6	108.7	126.9
Wt. gain/g. diet consumed (g.)	0.21	0.25	0.24	0.24	0.26
Carcass					
Wt. (g.)	109.2	134.4	133.5	130.5	148.1
Protein (%)	14.0	17.5	17.4	16.6	17.1
Fat (%)	17.3	20.4	20.2	18.5	20.7
Ash (%)	3.62	3.57	3.62	3.57	3.56
Liver					
Wt. (g.)	4.523	5.413	5.37	5.231	5.72
Protein (%)	14.5	18.5	17.8	17.6	17.8
Fat (%)	5.2	7.1	6.5	6.6	6.6
Blood					
Protein/100 ml. plasma	6.12	6.57	6.48	6.28	6.35
Hb/100 ml.	12.33	13.13	13.99	13.14	13.11
RBC Mil/cu.mm.	8.86	8.63	8.32	8.37	8.51
WBC/cu.mm.	6665	6555	6505	6572	6750



The effect of different antibiotics on liver storage of vitamin A, when the latter was fed at various levels, viz., 150, 250, 500 and 1,000 I.U./rat/week was also studied. The amount of vitamin A deposited in the livers showed practically no difference at 150 and 250 I.U. in the experimental and the control groups. At higher levels of vitamin A dosing (500 and 1,000 I.U.) more vitamin A was found in the liver of antibiotic fed animals. However, this difference is not significant when the values are expressed I.U./g. of the fresh tissue (Table 2). These observations do not confirm the findings earlier reported by Burgess *et al.*<sup>1</sup> and Coates<sup>2</sup>. So far as liver storage of vitamin A is concerned, the results of Hartsook *et al.*<sup>4</sup> are in agreement with ours. The dissimilarity observed between the present results and those of Hartsook *et al.*<sup>4</sup> in respect of production of vitamin A deficiency, could probably be explained on the differences in levels of dietary proteins and the antibiotics. The latter workers used a diet containing 22 per cent of casein and 100 mg. of aureomycin per kg. of the diet, while in the present study, all diets contained 10 per cent casein with 25 mg. of the antibiotics per kg. of the diets. Murray & Campbell<sup>10a,10b</sup> have studied the effect of aureomycin (66 mg./kg. diet) on vitamin A utilization in both male and female rats. Their results are quite variable. However, they demonstrated that aureomycin increased the potency of vitamin A in ovariectomised rats as judged by the vaginal smear assay. But they were unable to confirm the same by liver storage studies. On the other hand, the latter results support the present observations.

It is believed that the mechanism of conversion of carotene to vitamin A is enzymatic. However, it is not known whether bacterial enzyme plays any role in this mechanism or it is an independent enzyme system as other enzyme systems functioning in the gastro-intestinal tract. If any bacterial enzyme is involved in the transformation of carotene to vitamin A, then one would expect some alteration in the normal mechanism of vitamin A synthesis in the antibiotic treated animals, since antibiotics considerably modify the intestinal microflora. Coates<sup>2</sup> had found more efficient conversion of  $\beta$ -carotene to vitamin A in infected chicks treated with penicillin than untreated birds. However, the results of the present studies do not indicate any favourable action of antibiotics on the change of  $\beta$ -carotene to vitamin A, since the amounts of vitamin A in the livers of antibiotic treated and untreated normal rats, were almost identical. The increased rate of conversion observed by Coates<sup>2</sup> may probably be due to suppression of infection rather than due to the direct action of penicillin itself.

All the four antibiotics showed positive growth effect (Table 4 ; Fig. 1). This effect was not observable when vitamin A was absent from the diets, though antibiotics were present (Table 1). Similar observation was earlier reported by Swick *et al.*<sup>11</sup> showing that penicillin did not increase the growth of rats on diets marginal in vitamin A. Contrary to this, Murray & Campbell<sup>10b</sup> found aureomycin to increase the growth rate of rats which received no, or suboptimal amounts of vitamin A but had no effect when adequate amounts of vitamin A were fed.

The feeding of the antibiotics increased protein and fat percentage in both

carcass and liver of experimental animals. The increase in fat deposition is in good agreement with the findings of Hartsook and Johnson<sup>12</sup> and Teh Cheng & McCay<sup>13</sup>. These workers have, however, observed no difference in the protein content in antibiotics fed animals.

Since there was no change in the mineral content of antibiotics treated animals compared to the control, it seems that antibiotics do not have any effect on it. Teh Cheng & McCay<sup>13</sup> have reported a similar observation. The results on haematological values are in harmony with those of Mirone<sup>14</sup> showing that the antibiotics are without effect on blood constituents in normal rats.

#### ACKNOWLEDGMENT

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# Some Observations on the Effect of Asafoetida Oil on Intestinal Microflora

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**The effect of asafoetida oil on the intestinal microflora in rats fed on this condiment for long periods has been studied. The role of asafoetida as a food adjunct similar to antibiotics has also been studied.**

Spices and other aromatic substances which occupy an important place in dietary are generally considered as appetizers. Some of them, in addition, have also the reputation of possessing therapeutic properties, especially in the Ayurvedic system of medicine, for the treatment of intestinal disorders. But, their actual role in correcting these disorders is not known. Bose *et al.*<sup>1</sup> have reported that many of essential oils exhibit germicidal properties; asafoetida and other spices which are quite rich in these oils might therefore act in this way. This aspect has been investigated in these studies.

Bhat *et al.*<sup>2</sup> in an *in vitro* study on microbial fermentations found that asafoetida has no bacteriostatic properties but only disproportionates  $H_2/CO_2$  ratio. They suggested that the reputed carminative action of asafoetida may thus be due to this increase in  $H_2/CO_2$  ratio. As the conditions obtaining *in vitro* cannot be identical with those existing in the intestinal lumen, the effect of feeding asafoetida oil with a basal diet for long periods on the intestinal microflora has been examined; its effect *in vitro* on some of the isolated intestinal organisms has also been studied. Since previous reports<sup>3,4</sup> have indicated that the composition of the market samples of asafoetida varies very widely in respect of their oil, ash and resin contents, the oil of asafoetida was used in these investigations.

## EXPERIMENTAL

Asafoetida oil was obtained by petroleum ether extraction of a sample of Irani Khada<sup>4</sup>. The oil dissolved in groundnut oil was used in all the experiments.

Young weanling rats, divided into two groups according to sex and litter-mate, were fed with a synthetic diet (casein 10; groundnut oil, 10; cane sugar, 10; salt mixture, 2; vitaminized starch, 1 and corn starch, 67 parts) for a period of 8 weeks. During this period, the experimental group received



0.02 ml. of asafoetida oil (1 per cent solution in groundnut oil) per ounce per rat per day, and the control group an equal quantity of groundnut oil. At the end of the feeding period, the rats were anaesthetized and dissected. The ceca were removed aseptically. The contents of each cecum were squeezed out into tubes followed by washing down the cecum with 10 ml. distilled water into the corresponding tube. The contents of the tubes were vigorously shaken to homogenize the cecal matter, aided by glass heads. The homogenates at adequate dilutions were plated with different media. Eosinmethylene blue (EMB) agar for coliforms, tryptone-yeast extract-dextrose-BCP agar for acid formers and thioglycollate agar medium for anaerobes were used in the experiments. The counts were made after incubating the plates at 37°C. for 48 hr.

#### Effect of asafoetida oil on the growth of normal coliform flora of the cecum

0.02 ml. asafoetida oil in groundnut oil was added into one portion of the homogenized cecal contents and 0.02 ml. of groundnut oil into the other. Coliform counts were determined in both the flasks using EMB agar at the beginning and after incubation at 37°C. for 4 hr.

For *in vitro* studies, several isolations from EMB agar plates and from tryptone-dextrose agar plates (of the control group) were made and after a preliminary study, one strain of *Escherichia coli* and one of *Streptococcus faecalis* were selected for further work. In these experiments designed to study the growth of the two organisms in presence of the asafoetida oil, McConkey's broth was employed and the oil emulsified in 0.2 per cent bile salt solution was used. Twenty-four hour old cultures were inoculated into the McConkey's broth and incubated at 37°C. At different periods of

TABLE 1—EFFECT OF FEEDING ASAFOETIDA OIL ON INTESTINAL FLORA OF ALBINO RATS

	COLIFORM* × 10 <sup>4</sup>	ACID FORMERS* × 10 <sup>5</sup>	ANAEROBES* × 10 <sup>6</sup>
Control (receiving groundnut oil)	235	512	216
Experimental (receiving asafoetida oil)	78	725	112

\* Count per gram of cecal contents on fresh weight basis (average of 5 animals)

TABLE 2—EFFECT OF ASAFOETIDA OIL ON COLIFORM COUNT OF CECAL CONTENTS

	COLIFORM COUNT*	
	Control × 10 <sup>3</sup>	Experimental (0.02 ml. of the oil) × 10 <sup>3</sup>
Initial	4,900	4,000
After incubation for 3 hours at 37°C.	25,000	9,000

\* Count per gram of cecal matter on fresh weight basis



FIG. 1—PHOTOMICROGRAPH OF *Streptococcus faecalis* GROWN  
IN BILE SALT BROTH ( $\times 1000$ )





TABLE 3—EFFECT OF ASAFOETIDA OIL ON RATE OF MULTIPLICATION OF AN *E. COLI* STRAIN

INUCBATION TIME hr.	COUNT IN CONTROL PER ML. $\times 10^5$	COUNT IN EXPERI- MENTAL PER ML. $\times 10^5$
0	47	47
1	290	100
2	860	300
3	2,100	1,000
4	8,000	2,500
5	11,000	3,800
24	20,000	18,000

TABLE 4—EFFECT OF DIFFERENT CONCENTRATIONS OF ASAFOETIDA OIL ON RATE OF MULTIPLICATION OF *E. COLI* AND *S. FAECALIS*

CONCENTRATION OF THE OIL IN 5 ML. MEDIUM mg.	COUNT OF <i>E. coli</i>		COUNT OF <i>S. faecalis</i>	
	After 5 hr. $\times 10^5$	After 24 hr. $\times 10^5$	After 5 hr. $\times 10^5$	After 24 hr. $\times 10^5$
200	7	65	..	..
40	30	1296	..	..
20	210	1464	4	25
10	480	1674	17	180
5	456	1890	54	280
1	482	2460	78	320
0.5	462	2904	..	..
0.25	..	..	158	780
Nil (Control)	840	3046	315	1200

incubation, count of the organisms per ml. was determined by direct microscopic count method. The effect of concentration of the oil on growth of *E. coli* and *S. faecalis* was also studied by inoculating 24-hour old cultures into bile salt broth containing graded quantities of the oil emulsified in 0.2 per cent bile salt solution.

## RESULTS

The counts of microflora inhabiting the ceca of the control and experimental animals (Table 1) show that the coliforms and the anaerobes are much lower in number in the ceca of experimental animals than those in the control. The count of the acid formers, however, is higher in the experimental animals than in the control. In an *in vitro* study also, wherein the cecal contents are incubated with asafoetida oil, the number of coliforms had increased from 4900<sup>3</sup> to 25,000<sup>3</sup> (Table 2) in the control tube, but increased only from 4000<sup>3</sup> to 9000<sup>3</sup> in the experimental one. These data

clearly suggest that the asafoetida oil affects the growth of coliform bacteria. Results in Table 3 represent data obtained in a study of the effect of asafoetida oil on the rate of multiplication of one strain of coliform bacteria isolated from the cecum. The results indicate that the oil retards the multiplication rate. At the end of 24 hr., however, the count in both the control and experimental tubes is almost identical indicating that the oil in the concentrations studied is bacteriostatic for a short period.

Table 4 gives results on the effect of different concentrations of the oil on the multiplication of *E. coli* and *S. faecalis*. It is evident from the data that at concentrations higher than 20 mg./5 ml. the oil has pronounced inhibitory effect on the rate of multiplication of *E. coli*. For *S. faecalis*, however, the oil has a marked effect even at 5 mg. level.

With the latter organism an interesting phenomenon has been observed. At concentrations higher than 10 mg., the oil retards multiplication, and in addition, the cells show marked increase in size (compare Figs. 1 & 2). Subculturing of the swollen cells within 16 hr. after incubation into fresh medium restores their normal growth; but, after 24 hr. of incubation, they lose their viability and do not grow in a fresh medium.

#### DISCUSSION

The foregoing data demonstrate that the asafoetida oil, like an antibiotic, inhibits the growth of organisms. At low concentrations it seems to slow down the rate of multiplication. The *in situ* slowing down of the rate of multiplication of the coliforms may aid other organisms to multiply and increase in number. That is probably the reason for the observed increase in the number of the acid-forming bacteria in the experimental animals (Table 1).

Studies on the rate of increase in number of *S. faecalis* under the influence of the oil are of particular interest because the cells grown at 20 mg./5 ml. level of the oil assume giant sizes possibly due to an interference with cell division but not growth, when compared with the sizes of the cells grown in the control medium. Maximum size attained by such cells seems to bear a direct relationship with the concentration of the oil in the medium. At levels below 1 mg. the size of the cells in the experimental medium is the same as that of the cells in the control. The maximum size is attained at 20 mg. level, where the inhibition also is greatest. At intermediate levels, the sizes of the cells are in between that of the normal ones and those obtained at 20 mg. level. This abnormal feature has been observed by Gardner<sup>5</sup> as a consequence of penicillin toxicity on *Streptococcus faecalis* and has been referred to as swelling. The cause of this swelling has been shown to be due to an irreversible combination of penicillin<sup>6</sup> with some cellular component essential for division. Since the "swelling" observed in the present study is associated with a retardation of the multiplication of the cells, the effect of the oil may possibly be due to a similar mechanism. More work is, however, necessary to understand precisely the mechanism of action of the oil on the cells.

Thus, the results show that asafoetida shows an effect similar to that of antibiotics and this property rests in the oil portion. Many of the therapeutic

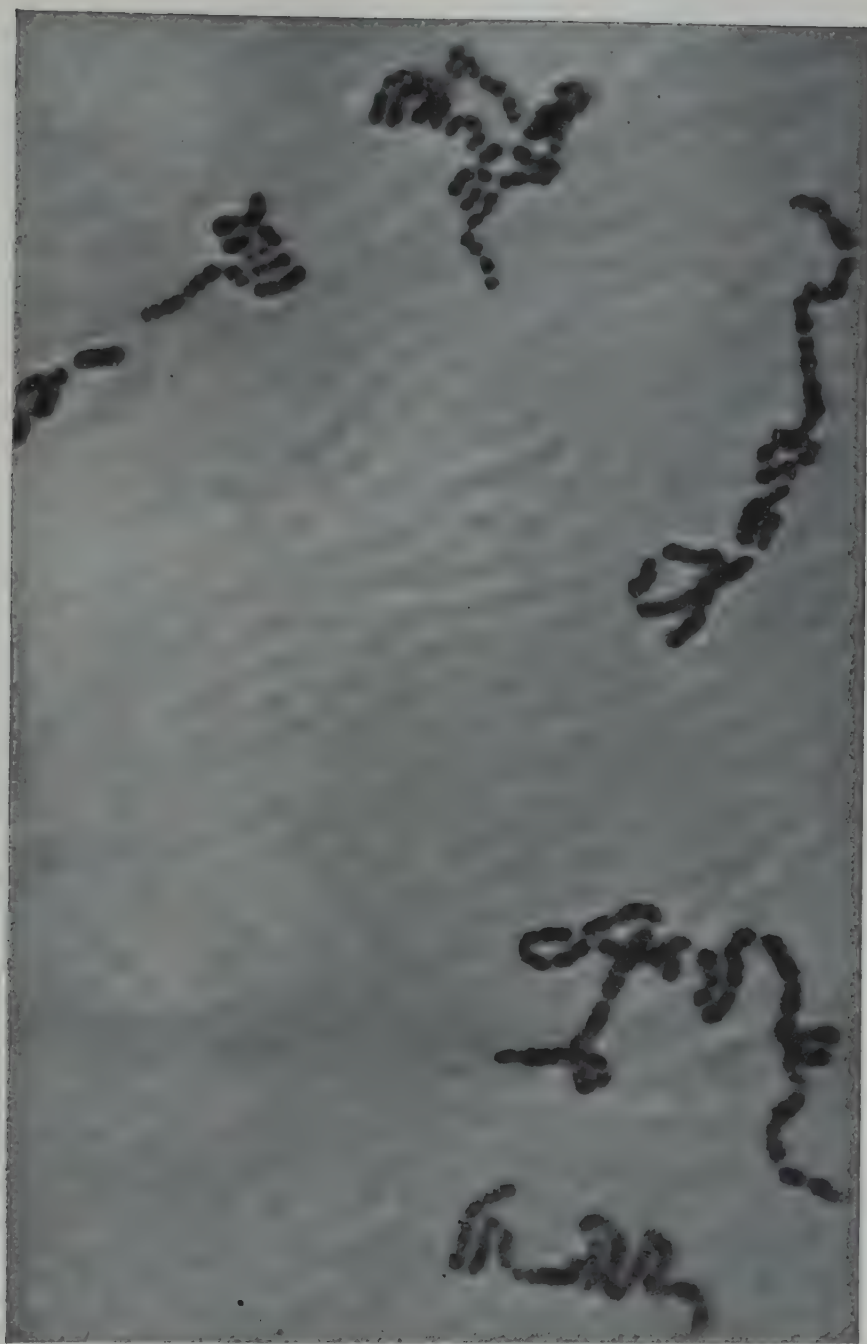


FIG. 2—PHOTOMICROGRAPH OF *Streptococcus faecalis* GROWN IN BILE SALT BROTH CONTAINING 20 MG. ASAFOETIDA OIL IN 5 ML. MEDIUM ( $\times 1000$ )





properties of this condiment may possibly be due to this antibiotic effect. More work is necessary especially on (1) the effect of asafoetida on the intestinal flora of human beings, and (2) its effect on other intestinal pathogens.

#### ACKNOWLEDGMENT

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# Metabolic Changes in Citrinin-resistant *Bacillus subtilis*

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The changes in amino acid and nucleic acid composition and in phosphatase activity of cells of *Bacillus subtilis* made resistant to citrinin have been studied. The development of resistance to citrinin in *B. subtilis* was associated with a change in the amino acid composition of the cellular proteins; the levels of histidine, lysine, proline, methionine, leucine, cystine and aspartic acid decreased while those of valine, phenylalanine and tyrosine tended to increase.

A similar comparative study of nucleic acid make-up revealed that resistance to citrinin is accompanied by increase in ribose nucleic acid content, desoxyribose nucleic acid remaining unchanged.

Resistance to citrinin resulted in reduced phosphatase activity in *B. subtilis* as well as in a shift in pH optimum to the acid side. This might possibly be due to alterations in cell permeability since phosphatases are known to be peripheral enzymes in micro-organisms.

A study of metabolic alterations attendant on the acquirement of resistance is of value in elucidating the mechanism of drug action. The present study was restricted to three lines of approach on the following basis using *Bacillus subtilis* as the test organism, since it is very sensitive to citrinin action and can be harvested with ease in sufficient amounts.

The observed inhibition by citrinin<sup>1</sup> of the assimilation of certain amino acids by *B. subtilis* suggested a possible alteration in protein content and amino acid composition of the bacterial cell material as a result of acquirement of resistance to citrinin. The known interference by antibiotics in nucleic acid metabolism<sup>2-4</sup> suggested a study of nucleic acid composition of normal and resistant strains of *B. subtilis*. Pratt and Duffrenoy<sup>5</sup> reported that the observed loss of nucleic acid (RNA) by diffusion into the surrounding medium in presence of terramycin indicated an impairment in the functioning of the cell membrane. Alterations in cell permeability, if any, can perhaps be best assessed by a study of peripheral enzymes in micro-organisms like phosphatases<sup>6</sup>. A comparative study of the phosphatase activity of normal and resistant strains was therefore included.

## EXPERIMENTAL AND RESULTS

### Development of resistance

Cells of *B. subtilis* were rendered resistant to citrinin by transplanting from



nutrient agar slants into sterile peptone water (peptone, 1 ; sodium chloride, 0.5 ; glucose, 1 per cent) tubes containing graded amounts of citrinin. The concentration of citrinin in each tube ranged from 1-5  $\mu\text{g.}/\text{ml.}$  increasing by 0.5  $\mu\text{g.}$  with each tube. The organism was transferred thrice in each dilution in order to effect better resistance.

### Determination of amino acid composition

Cells of the normal strain of *B. subtilis* were grown in 100 ml. lots of peptone water glucose medium in Roux bottles. The resistant cells were grown in presence of 5  $\mu\text{g.}/\text{ml.}$  citrinin in the same medium. After 24 hr. growth, the cells were harvested by centrifugation in the cold, washed twice in cold distilled water and after dilution in distilled water to give 20 mg. dry wt./ml. were subjected to acid hydrolysis as follows:

Cell material equivalent to 100 mg. dry wt. was suspended in 15 ml. of 6 N hydrochloric acid in a sealed tube, hydrolysed for 10 hr. at 15 lb. pressure and cooled. Two ml. of 2.5 M sodium acetate was added to the hydrolysates and the pH adjusted to 4.5. The hydrolysate was made to volume and filtered. A portion of the filtrate was shaken with ethyl ether to remove lipid material and pH of aliquot adjusted to 6.8 with sodium hydroxide and the volume was adjusted again.

The amino acids in the hydrolysates were estimated by the specific microbiological procedures using Cannon-Dispenser-Titration assembly<sup>7</sup> (Table 1).

TABLE 1—CITRININ RESISTANCE AND AMINO ACID MAKE-UP OF *B. SUBTILIS*

AMINO ACID	ASSAY ORGANISM	ASSAY RANGE $\mu\text{g.}$	PERCENTAGE OF CELL MATERIAL*	
			Normal strain	Resistant strain
<i>dl</i> -aspartic acid	<i>Leuconostoc mesenteroides</i> —P. 60 <sup>8</sup>	0-4	10.29	9.71
<i>l</i> -histidine	<i>L. mesenteroides</i> —P. 60 <sup>8</sup>	0-1	2.61	1.47
<i>dl</i> -lysine	<i>L. mesenteroides</i> —P. 60 <sup>8</sup>	0-16	18.34	17.72
<i>l</i> -proline	<i>L. mesenteroides</i> —P. 60 <sup>8</sup>	0-2	5.32	3.50
<i>dl</i> -methionine	<i>Lactobacillus fermenti</i> <sup>9</sup>	0-3.2	1.95	1.65
<i>l</i> -leucine	<i>Lactobacillus arabinosus</i> <sup>10</sup>	0-2.4	5.45	3.35
<i>dl</i> - $\beta$ -phenylalanine	<i>Leuconostoc mesenteroides</i> —P. 60 <sup>8</sup>	0-4	7.21	7.83
<i>dl</i> -valine	<i>Lactobacillus arabinosus</i> <sup>10</sup>	0-4.8	7.10	8.00
<i>l</i> -cystine	<i>Leuconostoc mesenteroides</i> —P. 60 <sup>9</sup>	0-1.4	1.51	1.02
<i>l</i> -tyrosine	<i>L. mesenteroides</i> —P. 60 <sup>9</sup>	0-2	2.29	2.95

\* Protein % of cell material (on dry basis nitrogen 6.25) were 76.2 and 73.1 respectively for the normal and resistant variants.

Note: Results expressed as *dl*-forms are expected to be half for the *l*-forms except for methionine.

TABLE 2—INFLUENCE OF CITRININ RESISTANCE ON NUCLEIC ACID MAKE-UP OF *B. SUBTILIS*

TYPE OF CELLS	NITROGEN CONTENT %	CELL MASS g./l.	RNA (%)		DNA (%)	
			20 hr.	42 hr.	20 hr.	42 hr.
Normal	12.2	0.750	9.70	7.35	2.89	2.73
Resistant	11.7	0.582	11.73	9.67	2.95	2.83

TABLE 3—CITRININ RESISTANCE AND PHOSPHATASE ACTIVITY OF *B. SUBTILIS*

SUBSTRATE*	PHOSPHATASE ACTIVITY (pH 8.4), $\mu$ G. INORGANIC PHOSPHORUS LIBERATED BY 10 MG. DRY WT. CELLS IN			
	1 HOUR		2 HOURS	
	Normal strain	Resistant strain	Normal strain	Resistant strain
Sodium- $\beta$ -glycerophosphate	90.8	52.0	168.0	112.0
Ribose nucleic acid	85.4	58.2	123.2	71.4
Desoxyribose nucleic acid	76.6	32.5	114.8	63.0

\* The reaction mixture contained 2 ml. of 5% Na- $\beta$ -glycerophosphate solution or 2 ml. of RNA or DNA solution containing 2 mg. of the compound.

TABLE 4—VARIATION IN PHOSPHATASE ACTIVITY OF NORMAL AND RESISTANT STRAINS OF *B. SUBTILIS*

(Substrate, Sodium- $\beta$ -glycerophosphate; Time of incubation, 1 hour)

TYPE OF CELL	PHOSPHATASE ACTIVITY* AT pH (OF THE REACTION MIXTURE)				
	7.2	7.8	8.4	9.0	9.6
Resistant	60.0	56.5	52.0	39.7	28.4
Normal	62.0	76.7	90.8	81.0	84.0

\*  $\mu$ G. inorganic phosphorus liberated by 10 mg. dry wt. cells/hr.

### Determination of nucleic acids

Normal and resistant cells of *B. subtilis* were grown in their respective media as before and were harvested after 24 and 42 hr. growth. Nucleic acids were extracted from aliquots of the cell suspensions by the procedure of Schneider<sup>11</sup>. The ribose nucleic acid (RNA) was determined by a modification<sup>12</sup> of the orcinol method of Albaum and Umbreit<sup>13</sup> and deoxyribose nucleic acid (DNA) by the diphenylamine method of Dische<sup>14</sup>.

The nitrogen content of the cell material was determined by a micro method of direct nesslerisation after digestion with phosphoric-sulphuric acids copper sulphate reagent<sup>15</sup> (Table 2).

### Determination of phosphatase activity

The phosphatase activity of normal and resistant variants of *B. subtilis* was determined at pH 8.4 using Na- $\beta$ -glycerophosphate, RNA and DNA as substrates (Table 3).

The system consisted of 3 ml. of 0.1 N veronal buffer at appropriate pH, 2 ml. of substrate and 2 ml. of cell-suspensions. After incubation at room temperature for the stated periods, the reaction was stopped by cold 10 per cent trichloroacetic acid solution and the inorganic phosphorus liberated was estimated by the modified Fiske-Subbarow method of Tausky and Shorr<sup>16</sup>.

The change in the alkaline phosphatase activity with pH in resistant variants was also followed in view of the report that the pH optimum for phosphatase activity of *B. subtilis*<sup>17</sup> varies with the environmental conditions under which the organism is grown. Comparative results obtained with normal and resistant strains are recorded in Table 4.

### DISCUSSION

Citrinin resistance alters amino acid make up of *B. subtilis* (Table 1). While there is a general decrease in the histidine, lysine, proline, methionine, leucine, cystine and aspartic acid contents in the resistant organism as compared to the normal strain, tyrosine, phenylalanine and valine contents increase. Apart from this, there is little decrease in the nitrogen content of the resistant organism. The lowered value for leucine in the resistant strain can be partly attributed to the inhibition *in vitro* by citrinin of its uptake by *B. subtilis*<sup>1</sup>.

Development of resistance to citrinin causes an increase in the synthesis of total nucleic acids and RNA by the micro-organisms. Smolens and Vogt<sup>18</sup> also reported that conditions inducing higher nucleic acid content are more favourable for the development of resistance in *Hemophilus pertussis*. According to Gale<sup>19</sup>, there is a decrease in RNA content with age in *Staphylococcus aureus* grown in presence of penicillin. The total nucleic acid content, as well as RNA decreases with age in both the normal and resistant variants of *B. subtilis*.

Resistance to citrinin results in reduced phosphatase activity in *B. subtilis* as well as a change in the pH optimum to the acid side. This may partly be due to alterations in cell permeability since phosphatases are known to be peripheral enzymes in *B. subtilis*.

### ACKNOWLEDGMENT

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# Studies on Antifungal Antibiotics : Metabolic Changes during the Production of an Antibiotic by *Bacillus subtilis*

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Studies on the metabolic changes during the production of an antibiotic by a strain of *Bacillus subtilis* indicate that three different phases may be recognized in the course of this fermentation. The first phase is characterized by low antibiotic production but rapid cell synthesis, oxygen uptake, glucose utilization and amino nitrogen consumption; the second, by enhanced rate of antibiotic production but slow cell synthesis, oxygen uptake and glucose utilization although amino acid consumption still continues, and the third by a slight decay in the antibiotic substance already formed. The rapid utilization of amino acids without the consequent cell synthesis in the second phase may be taken as an indication that amino acids are involved in the biosynthesis of the antibiotic molecule.

In an earlier communication<sup>1</sup> the optimum conditions for the production of an antifungal antibiotic by a strain of *Bacillus subtilis* have been reported. It is, now, felt necessary to investigate the biochemical changes that may occur during the fermentation process. Hendlin<sup>2</sup> studied the day to day change in pH, glucose and antibiotic concentration during the production of bacitracin—an antibacterial antibiotic by a strain of *B. subtilis*. An investigation of a similar nature was carried out by Shibasaki and Terui<sup>3</sup> with an antifungal antibiotic-producing strain of *B. subtilis*. This paper presents in some details metabolic changes particularly nitrogen metabolism during the production of an antibiotic by a different strain of *B. subtilis*. The strain AF<sub>1</sub> of *B. subtilis* used by Shibasaki and Terui was much longer (5-7 $\mu$ ) in length and the antibiotic formed had extremely low diffusibility through agar gel.

## EXPERIMENTAL

### Organisms

The antagonist was a strain of *B. subtilis* and the test organism, a strain of *Aspergillus niger* (G<sub>3</sub> Br).

### Medium

The production medium consisted of casein hydrolysate 1.0 per cent (w/v), beef extract 0.3 per cent (w/v) and glucose 1.0 per cent (w/v) and was adjusted at pH 7.2.

### **Fermentation experiment**

Fermentation was carried out by surface process. In a typical experiment 50 ml. of the medium taken in a 250 ml. Erlenmeyer flask were inoculated with 0.2 ml. of 2 days' old cell suspension<sup>1</sup> and incubated at 28°C. for different periods. Thereafter the contents of the flasks were analysed to indicate the change in composition.

### **Method of assay**

The antibiotic potencies of the broths after different periods of fermentation were determined by paper disc method<sup>4</sup> and the unit of activity was determined from the zone of inhibition (17.5 mm. in diam.) produced by 0.1 mg. of a standard preparation of an antibiotic, bacillomycin.

### **Method of determining growth of cells**

The weight of washed and dried cells was taken as a measure of cell growth, drying being carried out at 60°-70°C. for 24 hr.

### **Chemical analysis of the broths**

The concentration of glucose at different periods of fermentation was determined by Somogy's method<sup>5</sup> and the pH values with a Beckman glass electrode pH meter. Total nitrogen of the broths was determined by micro-Kjeldahl method, amino nitrogen by Sorensen's formol titration and ammonia nitrogen by the aeration method of Van Slyke. The cell nitrogen was calculated from the difference between the initial nitrogen of the broth and that left after the removal of cells.

### **Technique of determining oxygen uptake**

The oxygen uptake was followed manometrically by the same technique as adapted in Warburg's respirometer except that the respiration flask was replaced by a 250 ml. Erlenmeyer fermentation flask and that an empty flask of 1 litre capacity was interposed between the fermentation flask and the manometer to ensure adequate supply of oxygen. The fall in pressure due to carbon dioxide absorption was noted at constant volume as in Warburg's apparatus. In order to continue the observation over a period of five days incubation, the stop cock of the empty flask was released for re-entry of air every 24 hr. and then immediately closed. The daily uptake of oxygen was added up to get the total uptake during the period of observation.

## **RESULTS AND DISCUSSION**

The biochemical changes relating to pH, oxygen uptake, etc. during antibiotic production were studied. The results are given in Table 1 from which it appears that the rate of antibiotic production is extremely slow during the initial stages of growth when glucose utilization and oxygen uptake are fairly rapid. After two days of incubation the rate of antibiotic production is greatly accelerated particularly between the period 72 - 96 hr. till it attains a constant value on the fifth day, while cellular growth, oxygen uptake and glucose utilization during this period are progressively slowed down. The pH change



during the entire period is marked by a gradual fall from 7.2 to 6.71 till it records a slow but steady rise after the eighteenth hour of incubation.

### Nitrogen balance during antibiotic production

During the initial stages of fermentation when antibiotic formation is slow (Table 2), there is a rapid disappearance of amino and total nitrogen from the broth which, at the same time, records a rapid rise in its ammonia nitrogen content. Two days after, the antibiotic production is greatly enhanced when the disappearance of total and amino nitrogen from the broth still continues as before till the fourth day when elaboration of antibiotic nearly reaches its peak value. Ammonia formation, however, occurs all through the process although at a decreased rate from the sixth day.

TABLE 1—RATE OF ELABORATION OF ANTIBIOTIC IN RELATION TO pH, GLUCOSE UTILIZATION, GROWTH AND OXYGEN UPTAKE

PERIOD hr.	pH	GROWTH mg./l.	GLUCOSE CON- CENTRATION mg./ml.	ACTIVITY u./ml.	OXYGEN UPTAKE ml.
0	7.2	0.0	10.12	0.0	0.0
12	7.08	130	8.39	0.0	16.5
18	6.71	550	6.5	0.17	40.2
24	6.74	890	4.85	0.34	63.19
48	7.05	2930	3.7	1.19	106.30
72	7.46	3910	2.75	2.76	137.36
96	8.01	3982	1.8	4.0	150.61
120	8.3	3892	1.25	4.6	152.50
144	8.59	3550	0.84	4.6	152.50
168	8.71	3312	0.6	4.0	..

TABLE 2—NITROGEN BALANCE DURING THE PRODUCTION OF ANTIBIOTIC BY *B. SUBTILIS*

TIME hr.	TOTAL N <sub>2</sub> IN BROTH mg./10 ml.	AMINO N <sub>2</sub> IN BROTH mg./10 ml.	AMMONIA N <sub>2</sub> IN BROTH mg./10 ml.	CELL N <sub>2</sub> mg./10 ml.
0	16.240	6.44	0.840	0.0
12	16.086	6.30	1.512	0.154
18	15.596	5.60	1.736	0.644
24	15.204	5.04	2.10	1.036
48	12.964	3.64	2.94	3.276
72	11.872	2.10	3.36	4.368
96	11.816	1.26	3.50	4.424
120	11.928	1.40	3.64	4.312
144	12.320	1.54	3.78	3.920
168	12.572	1.96	3.85	3.668

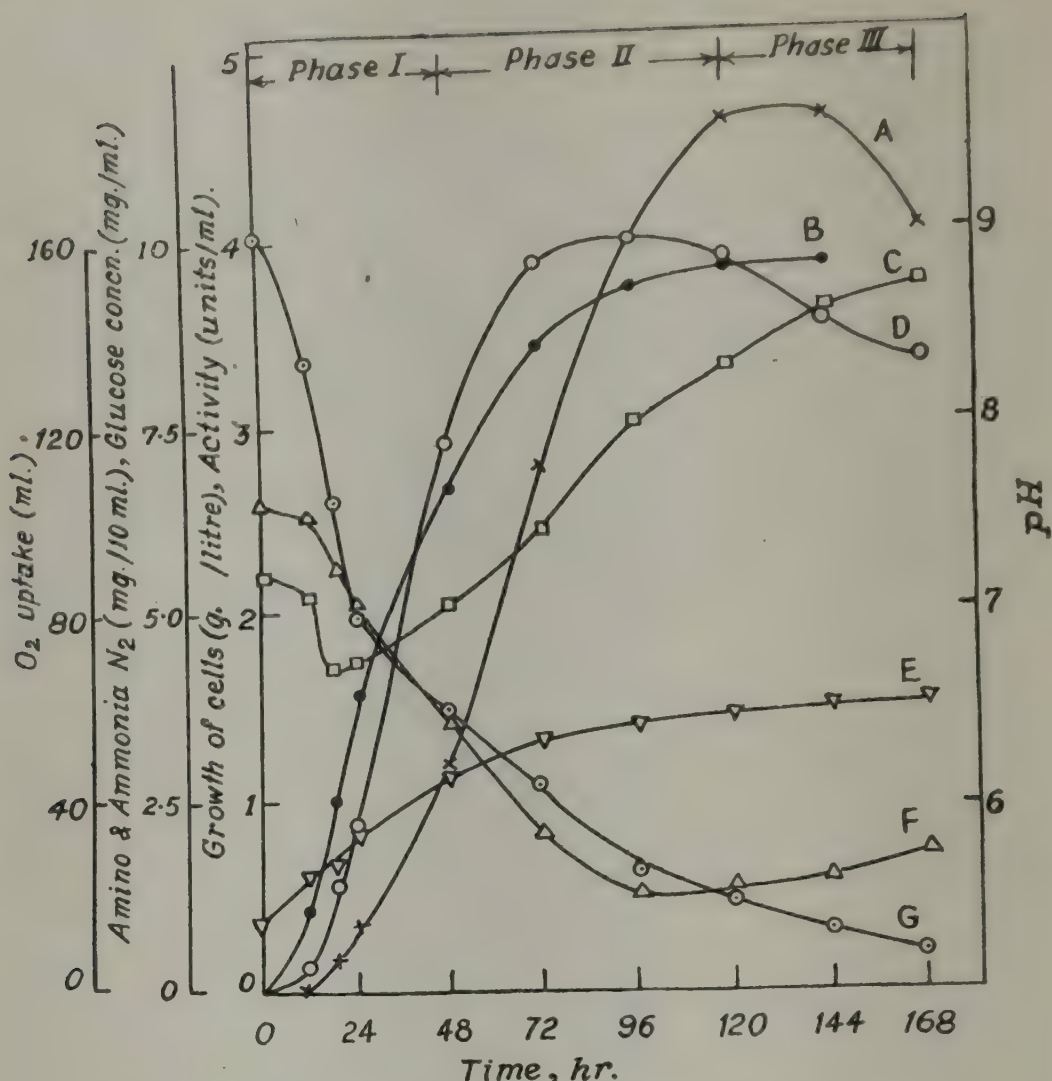


FIG. 1—METABOLIC CHANGES DURING FERMENTATION (A, ACTIVITY ; B, OXYGEN UPTAKE ; C, pH ; D, CELL GROWTH ; E, AMMONIA NITROGEN ; F, AMINO NITROGEN ; G, GLUCOSE CONCENTRATION)

It appears from the results (Tables 1 & 2) that this fermentation like penicillin fermentation can be divided into 3 distinct phases on the basis of the changes that may take place in the composition of the broths at different periods.

The first phase (Fig. 1) extending up to the second day of incubation shows rapid growth of cells. This may account for enhanced glucose utilization, rapid oxygen uptake to supply energy for cell synthesis, rapid consumption of amino nitrogen, etc. The initial fall in pH during the first phase despite continuous formation of ammonia may be due to formation of organic acids which may serve as intermediaries in glucose utilization. Shibasaki and Terui<sup>3</sup> also observed a higher rate of glucose utilization during the initial stages of antibiotic production by a strain AF<sub>1</sub> of *B. subtilis*. The second phase of fermentation extending up to the fifth day is marked by rapid antibiotic production and slow cell synthesis although the disappearance of amino and total nitrogen from the broth still continues. This may be taken as an evidence to show

that amino acids that disappear from the broth may be involved in the biosynthesis of the antibiotic molecule. In the third phase of fermentation commencing on the fifth day, fewer changes in the composition of the broths occur. This is perhaps due to ageing effect when metabolic activity is generally slowed down. This phase is, however, marked by a slight diminution in the concentration of the antibiotic due to degradation and also by a slight increase in amino and ammonia nitrogen of the broth due to autolysis of cells.

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#### Discussion

DR. K. GANAPATHI: What is the chemical nature of the antibiotic handled by the author?

DR. S. K. BOSE: It looks like a closed chain polypeptide.

DR. S. F. BOYCE: What is the evidence in favour of the suggested structure?

DR. S. K. BOSE: Work is in progress in this direction and the final conclusion can not be given at this stage.

DR. K. S. GOPALKRISHNAN: Which different fungi have been tested for anti-fungal activity and what is the zone of inhibition for unit activity.

DR. S. K. BOSE: *Aspergilli*, *penicillia*, and also some skin pathogenes were tested. The unit of activity is the zone of inhibition, 17.5 m. in diam. produced by 0.1 mg. of a standard preparation of bacillomycin.

DR. P. S. SARMA: Do any specific amino acids increase the yield of the antibiotic?

DR. S. K. BOSE: This aspect would form the subject matter of a second communication.



**Antibiotic Action and Bacterial Resistance.** M. S. KANVINDE (Ahmedabad).

*Med. Bull. (Gujarat & Saurashtra Provincial Br., Indian med. Ass.),* **1** (1955), 22.—Antibiotics inhibit bacterial cell growth by interfering with various metabolic processes of oxidation or synthesis or by altering cell wall structure.

Thus, streptomycin inhibits citric acid oxidation while penicillin, chloramphenicol, and tetracycline inhibit protein synthesis. The tetracyclines also inhibit citric acid oxidation. Tyrocidin destroys the semi-permeability of the cell wall by dissolving its lipoid. This action of tyrocidin also damages the host tissue cell wall and is therefore 'toxic' in effect. 'Nontoxic' effect of the other antibiotics is due to relatively decreased permeability of tissue cell wall to these antibiotics or absence of particular enzyme systems in tissue cells.

Capacity of bacteria to resist the action of antibiotics is brought about by a genetic change of mutation or by changes in the enzyme patterns to bypass a metabolic pathway inhibited by the antibiotic. Bacterial resistance may be (i) natural or (ii) acquired. The former is usually a genetic change and is permanent. The latter is a change in enzyme pattern and is slowly reversible if the antibiotic contact is removed. Organisms resistant to penicillin and tetracyclines belong to naturally resistant class. Resistance to streptomycin is usually acquired.

In a recent study of penicillin sensitivity of 61 strains of coagulase positive *Staphylococci* isolated in Sheth Vadilal Sarabhai General Hospital, Ahmedabad, nearly 80 per cent were found to be fully sensitive, 14.7 per cent slightly sensitive, and 4.9 per cent were resistant. The slightly sensitive and resistant strains were fully sensitive to aureomycin and terramycin.

Drug resistance is usually specific for a particular antibiotic or its closely related group. Use of combination of drugs may be rational in a mixed infection but should be judicious, e.g., bacteriostatic drugs like sulpha or tetracyclines should not be used with penicillin which is effective only on actively multiplying organisms. Synergistic action succeeds only where there is a tendency to drug fastness, e.g., with streptomycin where the resistant forms are inhibited by PAS. (*Abstract*)

## Recent Trends in Applications of Antibiotics

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**A review of applications of antibiotics in recent years has been given. The role of antibiotics in the case of disease and malnutrition and prevention of food spoilage has been discussed.**

Two scientific developments, above all others, have in this generation affected the course of mankind. One, of course, is nuclear fission, with all its unknown potential. The other, less talked about but of much greater direct benefit, is the discovery that lowly earth molds can help man in his everlasting fight against disease and malnutrition.

At a recent International Antibiotic Symposium, it was stated that some 3,500 different substances of varying potency and toxicity had been isolated and studied in the last 12 years. Of these, however, just over a dozen are clinically used.

After penicillin, streptomycin and chloramphenicol, came aureomycin (chlortetracycline) in 1948. Aureomycin was in a way, the first true broad-spectrum antibiotic. This was discovered by Dr. Benjamin Duggar, through a well organized screening programme conducted by the Lederle Laboratories Division of the American Cyanamid Company under the direction of the late Dr. Y. Subba Row. This was called a true broad-spectrum antibiotic, because it was found to be effective not only against Gram-positive and Gram-negative organisms, but also against some large viruses, rickettsiae and protozoa.

In 1950, came terramycin. Due to the close similarity in the behaviour of these two antibiotics, aureomycin and terramycin, a kinship was suspected between them for a long time and this was later confirmed when their chemical structure became known.

In 1953, arising out of a variety of circumstances and antibiotic research came the latest of the antibiotic drugs—achromycin (tetracycline).

Clinical trials and experiments over a long period before achromycin was placed in the market showed that this newest of antibiotics has fewer side reactions than the older ones, with greater stability as well. Patients who are sensitive to the older antibiotics react well in most cases to achromycin.

Quite apart from the role of antibiotics in the cure of diseases, is the importance they have achieved in the field of nutrition. The use of nutritive

substances such as vitamins, minerals and amino acids for therapeutic purposes has already been recorded ; but the use of therapeutic agents, the so-called antibiotics, for purposes of promoting growth and as diet supplements, is perhaps, without parallel in the history of medicine.

In combinations such as AUROFAC (aureomycin feed supplements) the diet-additive for livestock, aureomycin is boosting meat production by some 20 per cent above normal. The 20 per cent increase in the rate of growth of cattle, hogs and fowl and other livestock might well mean, directly or indirectly, the saving of similar percentage of human lives.

Exactly how antibiotics stimulate growth is not yet fully known, partly because all growth factors have not been isolated. The most obvious explanation of the effect, is that it is perhaps due to the action of antibiotics on the intestinal bacteria. The possible ways in which antibiotics may favourably affect the intestinal flora and hence promote growth, have been frequently mentioned since the original listing by Moore *et al.*<sup>1</sup>. The possibilities are as follows: (i) antibiotics may inhibit or destroy organisms which produce sub-clinical infections ; that is, they suppress organisms which produce toxic reactions and cause a slowing of growth of the host animal ; (ii) antibiotics may help in the proliferation or increase in activity of organisms which synthesize certain known or unknown vitamins and growth factors ; and (iii) antibiotics may inhibit organisms which compete with the host for the normally available nutrients. In support of these possibilities is the fact that a number of different antibiotics, having no special chemical properties in common, and exerting their anti-bacterial effects through different mechanisms, have a growth promoting effect. On the other hand, this growth promoting effect of antibiotics may diminish or disappear entirely when the animals are kept under clean and germ-free conditions<sup>2</sup>.

The growth promoting effects of certain antibiotics when injected may be due to their excretion into the intestines<sup>2</sup>. This has been shown to occur in calves under conditions where growth was improved following the injection of chlortetracycline<sup>3</sup>. Moreover, growth effect can be produced by certain antibiotics that do not readily enter the blood stream when given by mouth, e.g., streptomycin<sup>4</sup> and bacitracin<sup>5</sup>.

An apparent improvement in the nutritional status of animals may be often produced by adding small quantities of certain antibiotics to the diet. Utilization of food is often increased in growing animals by this addition as measured by a consequent decrease in the amount of food required to produce a unit gain in body weight. These effects are of great economic and practical importance<sup>6</sup>.

The prolonged and widespread feeding of antibiotics to farm animals has not led to deleterious results or to the establishment of resistant strains of pathogenic bacteria. There is much evidence which indicates that the premises may become "cleaned up" so that the growth of the resident animals is improved, even in the case of those animals from the same premises which are given unsupplemented diets<sup>6</sup>.

An approach to the study of the effects of antibiotics on the growth of



children presents certain difficulties as compared with experiments with animals, first, because every effort must be made with children to exclude the bacterial contamination that contributes so much to the antibiotic growth effect in animals and second, because of the slow rate of growth of human beings. The most marked effects of antibiotics on the growth of children may be expected where they are poorly developed due to subnormal diets or subacute intestinal diseases.

Several encouraging reports have appeared regarding premature infants. Perrini<sup>7</sup> fed chlortetracycline, 25 mg./kg. body weight, daily to 10 premature infants and found that they gained 8 per cent in weight during a ten-day period as compared with no weight increase in a group of 23 controls. Robinson<sup>8</sup> reported increased growth in premature infants receiving chlortetracycline who gained 29.5 g. daily as compared with controls who gained 18 g. daily. Five of the 15 controls died from intercurrent infections, while all of the supplemented group lived.

Lower mortality in a group of chlortetracycline treated premature infants together with increased growth was reported by Snelling and Johnson<sup>9</sup>; there was one death in a group of 47 who received 50 mg. of chlortetracycline daily as compared with 8 deaths in 48 controls. In a study of 57 supplemented premature infants and 56 controls, Coodin<sup>10</sup> found that oxytetracycline, 25 mg. daily failed to alter the rate of weight gain.

Scrimshaw *et al.*<sup>11</sup> studied Malayan Indian children, 7 to 12 years of age, living in the Guatemalan islands and subsisting on diets low in animal protein. In one trial, 14 children receiving 50 mg. of aureomycin daily for 15 months, showed a higher monthly height gain (0.48 cm.) than in 43 control children (0.33 cm.). Carter<sup>12</sup> carried out a prolonged investigation of a study of the effects of administering 75 mg. of aureomycin twice daily to crippled, mentally defective children for periods up to 3 years at the Florida Farm Colony. The average yearly gain in weight for the supplemented group was 6.5 lb. while the control group averaged 1.9 lb. in yearly weight gain. The supplemented group had a lower incidence of gastrointestinal disorders than the controls. There appeared to be no significant development of organisms resistant to aureomycin, for in all cases tried, the infection was controlled by increasing the dose of aureomycin to the full therapeutic level. In a carefully conducted study on healthy children from different institutions in Ceylon, de Silva<sup>13</sup> has shown that the administration of 50 mg. of aureomycin daily over a period of 6 months produced much greater increase in height and weight when compared with a similar group receiving placebos.

The effect of administering antibiotics to young males was studied by Haight and Pierce<sup>14</sup> at the Great Lakes Naval Training Station, where 330 recruits were selected at random into three treatment groups. For 7 weeks, each group received once daily a capsule containing 250 mg. of chlortetracycline in group 1. One hundred mg. of penicillin in group 2 and a placebo for group 3. After 7 weeks, the average gains in weight per man were 4.8 lb. for the aureomycin group, 4.1 lb. for the penicillin group and 2.7 lb. for the placebo group. Satoskar and Lewis<sup>15</sup> have shown that the administration

of small doses of antibiotics to healthy Indian students, led to a considerable and statistically significant rise in the level of plasma albumin along with a significant fall in the level of gamma globulin.

Another field in which antibiotics are of growing importance, is in extending the freshness of foods and preventing food spoilage. One of the prime instincts of man is to preserve food so that the extra supplies of today can be used for the needs of tomorrow. As our knowledge of food preservation has extended, it has been possible to have an ever increasing variety of seasonal foods, all the year around. Moreover, the improvements in the transportation system have also helped in bringing the more perishable foods to consumer with least delay. But in spite of this progress, there are many problems, most of which are concerned with preventing or retarding the process of bacterial spoilage in our foods. These spoilage problems are of the greatest economic importance for those foods where the consumer demands the quality of freshness, such as fish, poultry and red meats.

Fish after being caught, is subject to variable journeys depending on the tides and inclemencies of weather, much of the harm may be done before it even reaches the dockside. If bacterial growth and spoilage can be retarded aboard the fishing vessel, it means the trawlers can go out for longer periods and come back with larger catches of uniform quality and superior freshness. Many attempts have been made to retain the original freshness of fish by inhibiting the growth of spoilage micro-organisms. Formaldehyde and sodium nitrite have been tried but neither of these agents produced satisfactory results. Chlortetracycline has been found to inhibit a large variety of spoilage organisms and is extraordinarily effective even in extremely small concentrations.

In 1950, Tarr *et al.*<sup>16</sup> reported that chlortetracycline was an effective agent for maintaining freshness of fish. They repeatedly verified the remarkable effectiveness of chlortetracycline and stated that chlortetracycline was more effective in preserving foods than any of 14 other antibiotics studied<sup>17</sup>.

The application of 2 to 5 p.p.m. of chlortetracycline in ice is recommended for icing fish in various conditions. When a water solution of pure chlortetracycline freezes, the pure water freezes out first and the chlortetracycline forms a highly concentrated section in the centre of the frozen ice block. This concentration has been avoided in Acronice by the use of a product called ACRONIZE BI which combines chlortetracycline with a protective colloid and a metal salt. The net effect of this combination is that a fixed gelatinous network is formed throughout the solution. Chlortetracycline units are fixed in this network by means of the combined action of the metal salt.

Tarr *et al.*<sup>18</sup> have conducted several experiments on the comparative keeping quality of lingcod stored in ordinary ice and ices containing aureomycin. After 14 days in one of these experiments, fish iced with ordinary ice and 190 million bacteria per gram while those iced with ices containing 1 p.p.m. of aureomycin contained only 20 million.

In situations where it may not be practicable to obtain aureomycin, fish may be dipped in aureomycin solutions. Tarr *et al.*<sup>18</sup> have conducted experiments



on the keeping quality of red spring salmon stored in ordinary ice as compared with similar fish immersed in sea water containing 2 p.p.m. of aureomycin. They have shown that immersion in aureomycin solution effected a very striking improvement in keeping quality of the salmon. When aureomycin ice or solution containing 2 to 5 p.p.m. of aureomycin are used, the quantity of the antibiotic that may seep into the fish flesh would be extremely small, most of which again, is destroyed in the process of cooking.

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# Antibiotics in Raising Insects of Economic Importance with Special Reference to the Silk Worm *Bombyx mori* Linn.

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The effect of antibiotics, oxytetracycline, penicillin and carbomycin on the growth of chicks and baby pigs has been studied.

Chloromycetin and chlorotetracycline have been found to control successfully the flacherie disease of silk worms. The rate of growth of the worms is also increased and when supplemented with some amino acids, the yield of silk is enhanced.

Stockstad and Jukes<sup>1</sup> presented a paper at the Poultry Nutrition Conference at Chicago held in April 1949, embodying their significant observation that the growth rate of chicks was increased when fed with diets containing either chlorotetracycline or the fermentation residues of chlorotetracycline. This discovery led to an intensive study of the effect of other antibiotics on the growth rate of not only chicks but of other domestic animals, pigs<sup>2</sup>, lambs<sup>3</sup>, calves<sup>4</sup> and minks<sup>5</sup>.

The use of antibiotics as stimulants of animal growth, have now been so well established, that medicated feeds or feeds fortified with some antibiotic or other have come to be widely recognised in animal nutrition. During the last 4 or 5 years, there has been a steady increase in the employment of antibiotics as fortifying supplements to animal feeds. Some 500,000 pounds of these drugs are known to have been incorporated into feeds in the year 1954.

Antibiotics are administered generally at a level of 5 to 100 g. of the drug to a ton of the feed, depending upon the type of the antibiotic employed and the age of the animal treated. Tables 1 and 2 give data respectively pertaining to the growth of chicks and baby pigs when treated with different antibiotics<sup>7</sup>.

The mode of action of these antibiotics in stimulating the rate of growth of animals remains controversial but the studies point to the circumstance that the stimulation is in some manner connected with a marked change in the intestinal microflora. The antibiotics possibly condition the intestinal bacterial flora in favour of types which are capable of synthesizing growth factors. There is also a significant decrease in the mortality among the young

TABLE 1—GROWTH OF CHICKS

SUPPLEMENT	DOSAGE <i>g./ton</i>	AVERAGE WEIGHT OF 4 WEEKS OLD CHICKS <i>g.</i>	IMPROVEMENT OVER BASAL GROWTH %
None	..	269	..
Oxytetracycline	5	296	10
Penicillin	5	307	14
Carbomycin	2	274	2
Carbomycin	5	300	12

TABLE 2—GROWTH OF BABY PIGS

SUPPLEMENT	DOSAGE <i>g./ton</i>	AVERAGE DAILY GAIN <i>g.</i>	PER CENT INCREASE
None	..	0.642	..
Oxytetracycline	40	0.675	5
Carbomycin	40	0.759	18

TABLE 3—PERCENTAGE OF SURVIVALS AMONG BATCHES OF TREATED AND CONTROL SILK WORMS

UNINFECTED	ARTIFICIALLY INFECTED		
	No treatment	Chloromycetin	Chlorotetracycline
100	10-12	86-95	83-94
98	15-18	79-93	90-93
97	8-15	89-97	81-90
100	12-19	88-92	80-88

ones which are markedly free from intestinal disorders. In the case of poultry, medicated feeds (oxytetracycline) have been found to confer resistance to chicks against chronic respiratory diseases and against sinusitis<sup>8</sup>.

Generally it is during the earlier periods of development that the animal is most susceptible to these diseases and therefore needs to be prophylactically protected. Antibiotics serve this purpose admirably and help to tide over this critical period of infancy. Antibiotics are therefore useful in building up animal populations by reducing infant mortality among the young livestock.

While spectacular developments have been achieved in the field of raising mammals, antibiotics have so far not been tried for the development of fish and economically important insects. There is a heavy mortality among the fingerlings, a circumstance which constitutes the limiting factor in the rapid development of fisheries (Fisheries Officer, Madhya Bharat, Gwalior ; personal communication). Feeds fortified with antibiotics should be able to cause a reduc-

tion in the infant mortality among the fingerlings. This problem has recently been taken up by the Fisheries Department of Madhya Bharat.

Closely allied is the problem of raising populations of silk worms, on the success of which depends the output of silk. The silk worm is afflicted by two major diseases: Grasserie, which is known to be a polyhydral virus disease and Flacherie, a bacterial disease affecting the intestinal canal of the worm. Under certain conditions, these diseases have been found to occur in an epidemic form and are known to have caused serious losses to the industry. Thanks to the pioneering work of Pasteur, Pebrine, the protozoal disease of the silkworm has been effectively controlled.

Flacherie outbreaks have been successfully controlled by treating the feeds with either chloromycetin or chlorotetracycline. The disease can be artificially induced by infecting the worms with a spray of the bacterial suspension on the mulberry leaves. Table 3 gives the results of a set of four experiments, each batch consisting of 100 silk worms of the third and the fourth instars.

The results (Table 3) are highly eloquent ; the two antibiotics which have been tried are very effective in the control of this bacterial disease.

In addition to the effective control of disease, the antibiotics have been shown to potentiate an increase in the rate of growth by 10 to 12 per cent. When supplemented by amino acids, particularly glycine, alanine and serine, the yield of silk was enhanced by 12 to 14 per cent over the controls<sup>9</sup>.

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# Antibiotics : Their Therapeutic Uses and Hazards

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The importance of the discovery of antibiotics and their great role in the treatment and prevention of infections in general has been discussed. The difficulties with regard to individualization in dosage and the duration of treatment in particular cases have been given.

The importance of extending laboratory facilities in hospitals where antibiotics are widely used is stressed. Hazards in the use of antibiotics and their production of hypersensitivity in the body tissues and their direct toxic action is mentioned.

Since the discovery of anti-infectives like Prontosil by Dögmak and within a few years, of penicillin by Fleming, many compounds have been produced in the realm of anti-infectives and a host of antibiotics have been discovered ; one of them, chloramphenicol has even been synthesized. Had it not been for the hazards of the sulpha drugs, especially the earlier ones, such as sulpha-pyridine and sulphanilamide, the pace at which antibiotics are being offered for use might not have been so fast.

Improvement in bacteriological and pharmacological methods has enabled rapid study of these antibiotics as regards bacterial sensitivity and toxic effects.

The use of these agents has no doubt led to an overall control of the majority of infections caused by most bacteria, few of the fungi and some of the organisms smaller than the bacteria, such as the rickettsias. Infections caused by other fungi, most viruses and a few Gram-negative bacteria do not come under control as these are relatively insensitive to the antibiotics. It is known that actually some of the antibiotics are used to promote an easier growth of many of the viruses.

Although it is recognised that fever is the usual accompaniment of infection, it is often forgotten that fever may be due to causes other than bacterial invasion. It is, therefore, irrational to use antibiotics immediately fever is noticed unless the other symptoms and physical signs support the diagnosis of bacterial infection. It is important to remember that fever may accompany neoplasms, haematomas, leukaemia, etc. The proper way to use the antibiotics is to make a diagnosis, preferably bacteriological, whenever possible. In this connection it is important to know that simple smears (properly stained) of the sputum, urine, stools and of swabs taken from the throat, from pus in case of open sinuses, from wound surfaces and burn surfaces are a valuable

guide in a preliminary bacteriological diagnosis. The material should be so collected that a cultural examination is also possible from the same material.

The widespread use of antibiotics has made it imperative that simple methods of bacteriological diagnosis should be available and should be within the means of ordinary people. It is all the more important in hospitals and crowded places where cross infection by resistant bacteria has become common.

Except in certain fulminating infections where delay may be dangerous and antibiotics have to be used in large doses at once, it is advisable to watch the clinical picture, and, if possible arrive at a tentative diagnosis and then exhibit the appropriate antibiotic in adequate doses.

During the period of observation only symptomatic treatment should be exhibited. This attitude may raise a controversy but having come across many cases where, in an attempt to overcome the infection as quickly as possible, a series of agents is used one after another, starting with quinine injections, switching over to the sulpha group and quickly over to penicillin and other antibiotics one after another with the result that the correct diagnosis recedes to the background and is often never made. This would not be so serious if all the infections are controlled by this treatment but it is not so and often one is left with a hopelessly resistant and often undiagnosed infection, which progresses to a fatal ending.

In spite of abundant data having been made available by research workers regarding sensitivity of various bacteria to individual antibiotics, the dosage which is employed in actual practice is based more on habit and guess-work than on rational considerations. The mode of action, whether high bactericidal or only bacteriostatic, is also an important consideration. The bactericidal antibiotic acts best on young dividing forms and if the growth is slowed down by bacteriostatic drugs, the bactericidal action is often interfered with ; therefore, the combination of penicillin which is highly bactericidal with broad spectrum antibiotics which are mostly bacteriostatic may not be desirable in some cases. Literature on the combined use of antibiotics is confusing. In some infections such a combination has been found to retard progress and make the infection worse. In practice it is far too often forgotten that secondary infection may produce fever but the basic lesion may be quite different. This is often the case with lesions in the bronchii, gastro-intestinal tract, genito-urinary tract and the central nervous system. This is so in all lesions where superficial ulceration may occur such as carcinomas, lymphomas, collagen disorders, nutritional deficiencies and many others.

The treatment of the secondary infection may produce an apparent improvement but the actual disease progresses ; apparent improvement contributes to a delay in diagnosis and results in a fatal outcome, as the lesions have progressed beyond the stage where appropriate treatment would be effective.

It is also a common finding that congenital abnormalities or acquired lesions which cause obstruction to the free passage of discharges may, if not diagnosed and suitably treated, lead to a chronic intractable infection which may be associated with remissions but in which relapses invariably occur and the infection ultimately becomes intractable.



It cannot but be impressed that a complete diagnosis is essential in chronic febrile conditions caused by bacteria and anomalies, if found, treated surgically before a cure of the condition, even if caused by fully sensitive bacteria, can be expected.

Relapses often occur in chronic surgical infections after a preliminary improvement. The lesion may be on the skin or mucus membranes of the bronchii or gastro-intestinal tract. In all these areas there is a normal non-pathogenic bacterial flora. Pathogens have invaded the surface and the tissue beneath it after a trauma, such as by burns, superficial ulcerations or injury associated with other lesions. Antibiotic treatment clears some of these but the balance is upset, other bacteria, perhaps more resistant, start growing there and the infection continues this time by other organisms not sensitive to the antibiotic being used. It is essential, therefore, that repeated bacteriological examination of such lesions such as swabs from ulcers, discharge from sinuses, sputum, faeces, urine, etc. be made. One is often rewarded by finding a different bacterial flora which may then lead to a change to the appropriate antibiotics and a better control of the infection. In such studies, it is not uncommon to find that highly resistant bacteria are growing such as *Bacillus pyocyaneus*, *Bacillus prodigiosus* *Staphylococcus pyogenes* and entirely different antibiotics, such as polymixin B, bacitracin, neomycin, may be necessary in such cases in spite of their greater toxicity.

Before going to some of the details regarding dosage of individual antibiotics and hazards associated with their use, it is necessary to remember that: (i) antibiotics have revolutionized the treatment of infections, the vast majority of these have been brought under control; (ii) this advancement has brought a far greater necessity of a careful diagnosis before commencing the treatment; (iii) simple bacteriological methods such as smear examinations, simple culture media for preliminary inoculation of material must be freely and widely made available; (iv) a considerable expansion of the available laboratory facilities in hospitals and bigger centres is essential for better results and for avoidance of intractable complications; (v) the deliberate use of single antibiotics in adequate dosage is favoured except in those cases where synergistic action is proved and where a delaying action is not likely to occur; (vi) Restriction is necessary on the exaggerated claims by pharmaceutical firms for their products mainly based on *in vitro* studies and experimentally induced *in vivo* infections. Such claims far from simplifying the treatment confuse the medical man and encourage ill-considered changes from antibiotic to antibiotic or to incongruous combinations; (vii) the medical practitioner must ask himself: Has he made the diagnosis reasonably certain? If not, how can he do so? Should he withhold the antibiotic till the picture is clear? Must he use them at once as the infection is severe? Teaching the medical student in the wards must be influenced by these considerations.

### Dosage

This is based on *in vitro* and *in vivo* experiments. In the case of penicillin the dose first advocated was the minimum effective one.



Penicillin has still maintained its primary place as the most useful and the least toxic of the antibiotics even when used in enormous doses in resistant and severe infections.

Streptomycin is more toxic, induces quick resistance in most bacteria that are originally sensitive to it and must be used in adequate dose for short periods in all infections except those caused by the tubercle bacillus and even here in combination with other drugs such as PAS and INH.

As regards the wide spectrum antibiotics, it is extremely difficult in practice to determine the dose required for an individual patient. The dosages originally recommended, e.g., chloramphenicol in typhoid, were apparently too high and toxic at least for some patients, same was the case with chlor- and oxy-tetracycline. Subsequently, the doses recommended were reduced to one gram in 24 hr. for the latter two for the average adult in moderately severe infections.

More work requires to be done in the matter of dosage with respect to the wide spectrum antibiotics. As regards parental preparations of these, the toxicity is stressed more and minimum doses advocated. It is difficult to determine these and as the parental method of administration is advocated in serious and heavy infections and as fatality in these is still high, it is difficult to distinguish the toxic manifestations of the drug from those of the infection.

### **Duration of treatment**

To determine the duration of treatment with penicillin is usually not difficult, about a week more after the infection is controlled except in infective endocarditis and some deep seated infections in which case it may have to be administered for a longer period, the exact period is then difficult to define.

In the case of wide spectrum antibiotics, it is still more difficult to determine the duration of treatment in many infections. This is largely due to the fact that they are bacterio-static drugs and the body defences have to be active to control the infection completely.

This difficulty is best exemplified in the use of chloramphenicol in enteric fevers. Relapses are quite common although the control is fairly quick. It is not possible to determine the optimum dosage and its duration in a given case. Similar is the case with other antibiotic achromycin, tetracycline and erythromycin.

### **Choice of antibiotic**

In the majority of infections penicillin either alone or combined with streptomycin is the treatment of choice. In spite of the availability of painless long acting preparations, crystalline penicillin G in an aqueous solution is preferred and is exhibited in an adequate dose at least twice daily. More frequent administration and much larger doses will be required in exceptionally heavy and bacteraemic infections, e.g., bacterial endocarditis, pneumococcal meningitis, etc. It is questionable whether preparations with procaine and also depot preparations should be advocated except in rare instances.

## Chloramphenicol

It is the antibiotic for the treatment of enteric fever. The dosage schedules vary with the physicians. The high dosage originally recommended has been universally given up because of dangerous reactions associated with them. The optimum dosage and duration of treatment, as has been mentioned before, have still to be worked out.

The broad spectrum antibiotics oxy- and chlorotetracycline and tetracycline itself, chloramphenicol and erythromycin require to be used with great care and discernment. Their spectra are wide but several organisms are already getting resistant to them.

Erythromycin is often extremely valuable in controlling penicillin resistant Grampositive infections and even more so in restoring the normal flora of the gastro-intestinal tract. But bacteria may become resistant to erythromycin also. The use of wide spectrum antibiotics is often like a game of chess, wise moves are required for good control. They are difficult and if wrong moves are made a deadlock is often reached.

Polymixin B, bacitracin and neomycin must be considered if such a deadlock is reached with wide spectrum antibiotics. An attempt, however, should always be made to get the bacteriological diagnosis in such cases.

## Hazards

The majority of complications of antibiotics therapy can be attributed to: (i) the development of sensitisation causing allergic manifestations and peripheral vascular shock of an anaphylactic nature; (ii) direct toxic action of the drug; (iii) disappearance of sensitive organisms and introduction of other resistant pathogens; (iv) change of normal balanced flora of skin and mucus membranes, and substitution by pathogenic organisms, e.g., *Staphylococcus aureus* in the gastro-intestinal tract, *Pseudomonas pyocyaneus* in the genito-urinary tract or in superficial wounds; and (v) manifestations of vitamin deficiencies as bacteria required for their manufacture may be destroyed.

Sensitisation may occur to any of the antibiotics but appears to be commonest after penicillin and streptomycin. Fever, manifestations on the skin e.g., erythema or urticaria are common. Erythema multiforme and exfoliative dermatitis may be serious and sometimes fatal.

The drug must be stopped and the allergy treated by immediate use of anti-histamines and or sometimes by cortisone and ACTH.

Peripheral shock with vaso-motor and respiratory distress sometimes occur and might prove fatal. Several such cases have been recorded after the use of procaine penicillin and crystalline penicillin. Prompt treatment for peripheral failure such as plasma, nor-adrenaline and ACTH or cortisone may tide the patient over. The important point is that one should be prepared and have the emergency measures ready on call.

## Toxic symptoms

Also, direct gastro-intestinal irritation used to be frequent with chlor- and



oxy-tetracycline. Individuals vary considerably in their tolerance to these drugs.

Nausea, vomiting and/or diarrhoea sometimes occurred with 250 mg. 6-hourly or did not occur even with 500 mg. Some persons feel weak for a few days even after the infection is controlled.

Allergic manifestations are rare. Tetracycline showed toxic effects only occasionally.

Erythromycin has been on the whole well tolerated.

Renal irritation, i.e., albuminuria and casts in the urine have been frequently reported, after streptomycin, bacitracin and polymixin B. However, these drugs are required in serious and resistant infections and should be used.

The really difficult hazard is the occurrence of superinfections with broad spectrum antibiotics. If not specifically and repeatedly looked for they are often missed and when ultimately discovered, it may be too late for effective treatment.

Somewhat similar is a change in the normal flora, which is usually well balanced and non-pathogenic, to an unbalanced one. This may or may not be associated with an infection. Usually, however, some resistant pathogens ultimately take hold and produce a serious complication. This is extremely difficult to diagnose unless good laboratory facilities are available for bacteriological examinations.

While acknowledging the great contribution made by antibiotics in the treatment of disease, it is necessary to point out that more serious study of the natural history of infection is necessary, great care is required in the selection of the antibiotic prior to use, and a constant watch is necessary to diagnose and deal promptly with complications and superinfections.

Also it is necessary to keep in mind that the cost of most of the antibiotics is still prohibitive for the average individual and has to be kept in mind while prescribing them. They should certainly not be used for all and sundry cases of fever.

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### Discussion

DR. K. GANAPATHI: Will you recommend the many types of antibiotic combinations as are frequently seen in the market, except such well established combinations as streptomycin-PAS, streptomycin-isoniazid?

DR. B. B. YODH: It is a trial and error business. The proper combinations of bacteriostatic antibiotics with a bactericidal one need careful biological control. Combination is not advisable in the majority of cases. Combination should be tried only when one has failed with the use of single antibiotics. When mixed infection has been definitely established, the combination can be used.

DR. V. N. KRISHNAMURTHY: Streptomycin used as a synergistic with tetracycline has given good results on streptococcus, staphylococcus, *Bacterium coli* and *Bacillus pyocyaneus*. Hence the combination may be used with advantage in cases of mixed infections such as pulmonary suppuration.

# Personal Experiences with Tetracycline and Carbomycin : Part I—Carbomycin in Kala-azar

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Reference is given to the literature suggesting the need for trying carbomycin in the treatment of kala-azar with the conclusion that in the doses employed carbomycin is not of value in the treatment of kala-azar.

The suggestion for trying carbomycin in the treatment of kala-azar was taken up on the basis of the following two extracts from the literature:

"Of particular interest is the effect of erythromycin and of magnamycin on trypanosomes and on *Leishmania donovani*. Magnamycin at a concentration of 125 mg./ml. inhibits *L. donovani* *in vitro*.

"Achromycin, another metabolic product of *Streptomyces* is also active against trypanosomes and is similar in anti-bacterial spectrum to erythromycin and carbomycin.

"Erythromycin, carbomycin and achromycin, because of their lower toxicity, may be useful substitutes for the organic arsenicals and antimonials now used for treatment of trypanosomes and leishmaniasis".

"The effect of carbomycin on both protozoa and spermatozoa has been studied by Seneca and Ides. In these *in vitro* studies carbomycin was shown to inhibit cultures of *Entamoeba histolytica* in concentrations of 31.25 mg. to 125 mg. per ml. *Trypanosoma cruzi* and *Leishmania donovanai* were inhibited in concentrations of 31.2 to 62.5 mg. per ml., while *T. rhodesiensi*, *T. vaginalis* and *T. fetus* were inhibited by concentrations of 125 to 250 mg. per ml. It required 44 minutes at a concentration of 10 mg. per ml. to immobilize human spermatozoa. These authors feel that *in vitro* studies indicate that a clinical trial should be made using carbomycin in the treatment of amoebiasis, leishmaniasis and trypanosomiasis<sup>2</sup>".

Carbomycin was used in 16 cases of kala-azar, the initial diagnosis in fifteen of which was established by the demonstration of the L-D bodies in sternal puncture or spleen puncture. In one case in which this was not done, the sternal puncture was negative and the patient's general condition did not permit a spleen puncture. But the indirect tests for kala-azar were all positive and the clinical picture was absolutely typical.

Eight cases were given the full course of treatment on the following schedule: 2 tablets of 250 mg. each every six hr. for the first four days and then one tablet every six hr. for 12 days, making a total of 80 tablets in 16 days. The treatment in the other 8 cases was for a shorter period: Table 1 summarizes all the case records. This was because of the patients being very ill, showing no response in the first few days of treatment, the antimony treatment having had to be started as a life-saving measure.

TABLE 1—CARBOMYCIN (MAGNAMYCIN) IN KALA-AZAR

CASE NO.	NO. OF DAYS TREATMENT	TABLETS GIVEN	TEMPERATURE (°F.)		SPLEEN: FROM COSTAL MARGIN (in.)		LIVER (in.)		L.D. BODIES IN STERNAL PUNCTURE OR SPLEEN PUNCTURE	
			Before	After	Before	After	Before	After	Before	After
1.	16	80	98.4°-100°	Normal	5½	4½	4½	4½	++	+
2.	16	80	98.4°-101°	98.4°-100°	5½	8	2	2	++	++
3.	16	80	98.4°-100°	98.4°-99°	3	3	1¼	1¼	++	+
4.	16	80	98.4°-101°	Afebrile from second day	5¼	5¼	2	2	++	++
5.	2	14	102°-103°	102°-103°	5¼	5¼	1½	1½	++	Not repeated
6.	(a) 8	20	98.4°-99°	(a) 98.4°-103°	5	5	2	2	++	Not repeated
	(b) 2	4		(b) 98.4°-103°						
7.	3	25	99°-104°	99°-102°	4	4	1½	1½	+	Not repeated
8.	10	56	100°-103°	98.4°-101°	7½	7½	2	2	++	Not repeated
9.	16	80	99°-102°	Afebrile	5	5	1½	1½	+	Not repeated
10.	5	20	98.4°-103°	98.4°-102°	3½	3½	1	1	++	Not repeated
11.	9	52	101°-104°	99°-102°	3¾	3¾	1	1	+	Not repeated
12.	3	24	98.4°-101°	98.4°-100.5°	3½	3½	2½	2½	++	Not repeated
13.	16	80	100°-103°	98.4°	2½	1½	1½	1	Negative	Not repeated
14.	16	80	98.4°-99.5°	Normal	4	4	1¾	2	++	Not repeated
15.	16	80	99°-101°	Normal	5½	5½	5¾	5½	++	Not repeated
16.	8	48	98.4°-100°	Normal	2	2	1	1	+	Not repeated

++ indicate the number of L.D. bodies found in the puncture material.



Case No. 6 was particularly interesting in that the administration of carbomycin was associated with a deterioration in the fever and other general features of the illness. When carbomycin was stopped, the condition appeared to have improved and when again after five days, the drug was resumed, the temperature and pulse rate went up. This patient was ultimately cured with antimony. Except for the unusual febrile reaction in this patient and diarrhoea in another patient, there were no side-effects worth mentioning.

In 7 out of the 16 patients the temperature showed a reduction compared to the pre-treatment temperature. The temperature is one of the least dependable signs in kala-azar often showing improvement with rest alone. However, only in two cases was there a slight reduction in the size of the spleen and liver. In all the other 14 cases there was no alteration. The total white blood corpuscle (W.B.C.) count was low in all the cases at the commencement of treatment. In 6 cases there was a significant rise in the W.B.C. count at the end of treatment. In 4 cases it was less and in the remaining 6 cases it was the same. The reversal in the albumin-globulin ratio noted in 13 of the cases did not show any appreciable change at the end of treatment.

At the end of treatment, the sternal puncture was repeated only in 4 cases as the others would not permit a repetition of the procedure. In all the four cases L-D bodies were present.

Except in 2 patients who got discharged against advice, antimony treatment was given on the usual schedule for the remaining 14 cases with the anticipated improvement in their clinical state.

### CONCLUSION

The following conclusions seem reasonable on the basis of the above study: Results achieved *in vitro* may not necessarily be always applicable to the living body. There may be a variation in the strains of *Leishmania donovani* used in the experiments of Seneca and Ides and those causing the disease in South India. In the doses employed in the present study carbomycin is not of value in the treatment of kala-azar. Whether larger doses administered over longer periods of time can produce any appreciable results may require further study.

### ACKNOWLEDGMENT

We are indebted to Chas. Pfizer & Co., for the supply of magnamycin, their brand of carbomycin, used in these studies.

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# Personal Experiences with Tetracycline and Carbomycin : Part II—Tetracycline in Broncho-pulmonary Suppuration

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**Rationale of intense medical treatment in acute lung abscess is discussed. The place of tetracycline in the management of 11 cases of broncho-pulmonary suppuration is evaluated.**

During the decade 1940-50 it was held by most authorities on chest diseases that both acute lung abscess and bronchiectasis were essentially surgical diseases with medical care only during the period of preparation. Since then, however, there are a number of reports of medical treatment of acute lung abscess resulting in a complete cure clinically and radiologically. Penicillin, penicillin with sulphadiazine, chlortetracycline, oxytetracycline and chloramphenicol have all been studied by various workers<sup>1-3</sup>. We have ourselves succeeded in producing such cures with penicillin. One of the earliest cases treated on the usual massive dosage schedule in 1948 on examination by X-ray recently showed no lesion at all in the left upper lobe which was the site of the abscess.

In an essentially acute inflammatory process such as lung abscess, the adequate and timely control of the causative organisms can undoubtedly lead to complete healing. The idea that an acute lung abscess is essentially a surgical problem needs revision.

With regard to bronchiectasis of the irreversible type, however, the antibiotics undoubtedly play an important role in the control of the recurrent episodes of infection giving rise to fever, cough, expectoration, hemoptysis and lassitude. It is extremely doubtful whether the anatomical abnormality can be fully cured by chemotherapy alone. But even here, the concluding paragraph of a recent editorial<sup>4</sup> in the *Lancet* reproduced below, is suggestive of a changing concept:

‘Evidence is accumulating that in the great majority of cases modern medical treatment, with its emphasis on postural drainage and the use of antibiotics, greatly limits the disability from this disease. The cases most in need of surgery are usually the least likely to benefit from it on account of the extent of the disease or poor general condition. Despite the excellent results of many operations, there are good grounds for a conservative approach to the treatment of bronchiectasis’.

TABLE 1—TETRACYCLINE IN BRONCHO-PULMONARY SUPPURATION

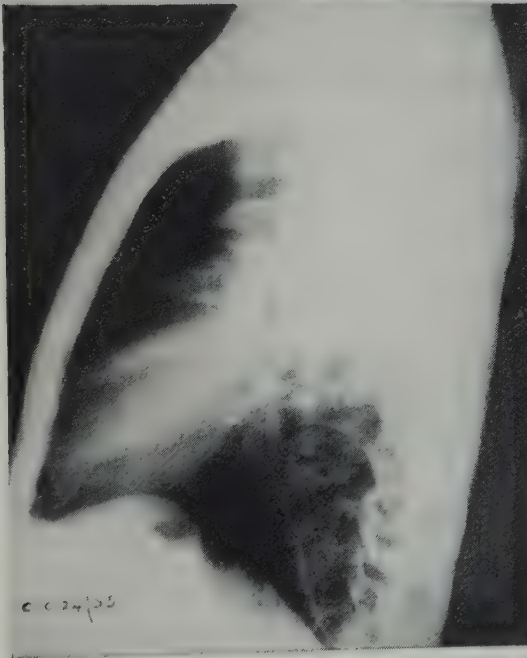
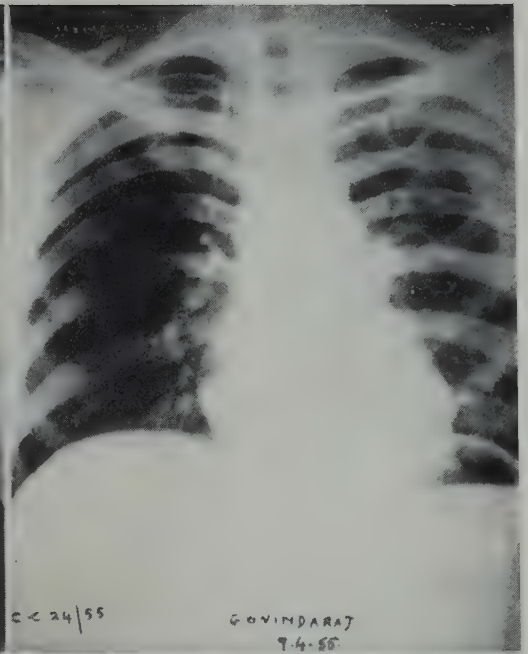
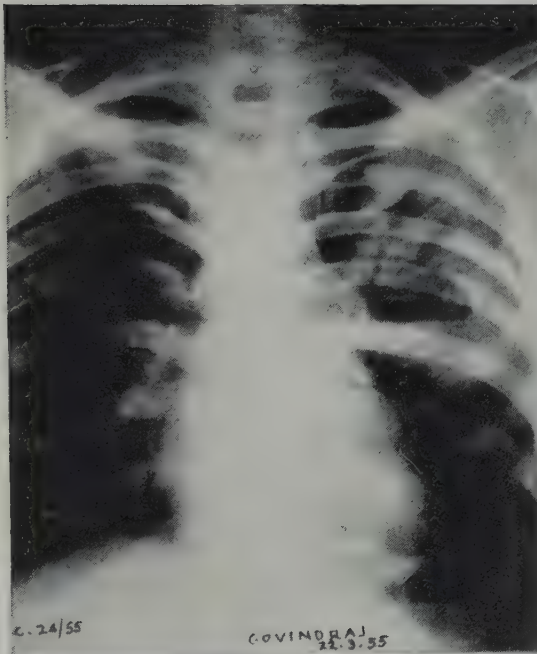
CASE NO.	SEX	AGE YEARS	DIAGNOSIS	DURATION OF SYMPTOMS	No. OF CAPSULES GIVEN	TEMPERATURE (°F.)		COUGH		PHYSICAL SIGNS*		SPUTUM QUANTITY oz.		ORGANISMS CULTURED
						Before	After	Before	After	Before	After	Before	After	
1.	M	30	Abscess, L.U.	10 days	104	N	N	+	+	+	+	7	Nil	Str.
2.	F	17	Abscess, dorsal segment, L.L.	9 days	158	N	N	+	+	+	+	10	1	Str., Pn.
3.	M	25	Abscess, L.U.	8 months	250	N	N	+	+	+	+	8	2	Str., Sta
4.	M	63	Abscess, L.U.	2 months	120	104	(4th day)	+	+	+	+	6	Nil	Sta., & B. faecalis
5.	F	13	Bronchiectasis Left Lower	2 years	40	N	N	+	+	+	+	4	1	..
6.	M	35	Bronchiectasis Both Lower	4 years	206	102	N	+	+	+	+	8	5	Str., Pn.
7.	M	40	Bronchiectasis Left Lower	3 months	184	N	N	+	+	+	+	21	3	Str., B. coli
8.	M	20	Bronchiectasis Left Lower	2 years	103	N	N	+	+	+	+	14	4	Str., B. coli
9.	M	29	Bronchiectasis Right Middle	5 months	108	101	N	+	+	+	+	10	2	Str., Pn.
10.	M	28	Bronchiectasis Left Lower	6 months	37	N	N	+	+	+	+	10	3	..
11.	M	29	Bronchiectasis	6 months	44	N	N	+	+	+	+	8	1	Str.

M, male; F, female; N, normal; S, slight; Str., Streptococci; Sta., Staphylococci; Pn., Pneumococci; +, +, + refer to the severity of the cough and physical signs; \* Evidence of disease found by examination.



(a)

(b)



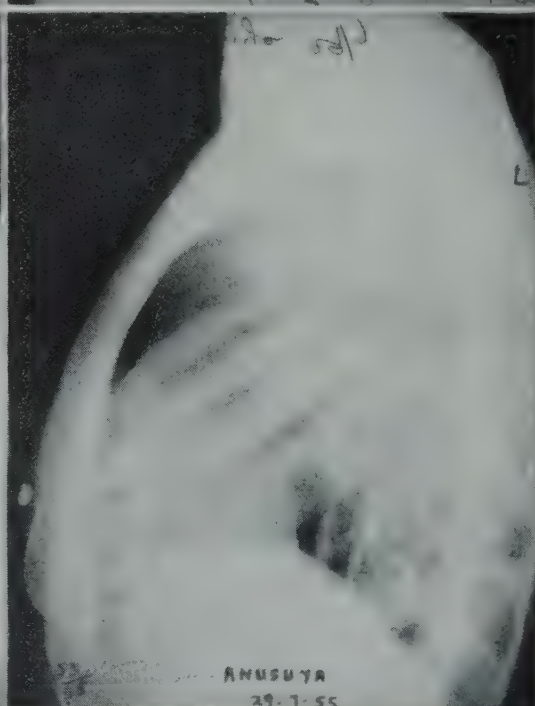
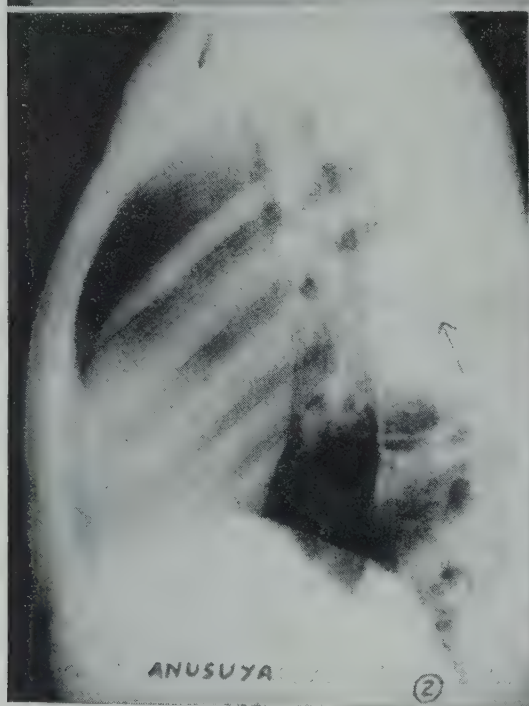
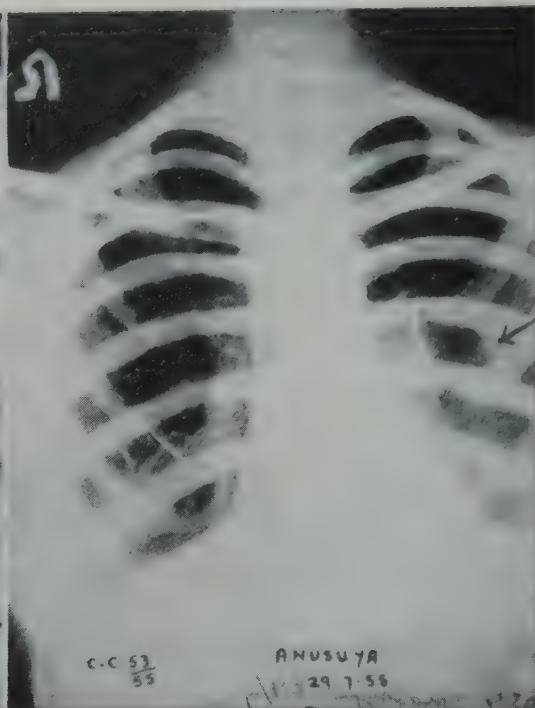
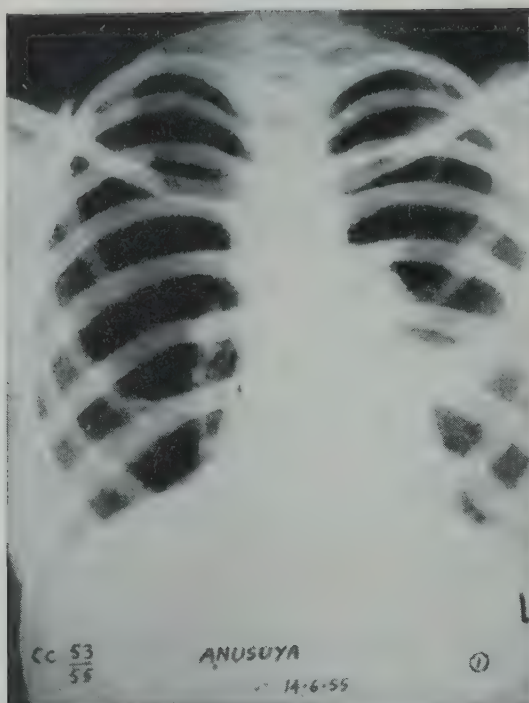
(c)

(d)

FIG. 1—ABSCESS LUNG, LEFT UPPER LOBE (a) PA VIEW BEFORE TREATMENT ;  
(b) LEFT LATERAL VIEW BEFORE TREATMENT ; (c) PA VIEW AFTER TREATMENT ;  
(d) LEFT LATERAL VIEW AFTER TREATMENT

(a)

(b)



(c)

(d)

FIG. 2—ABSCCESS LUNG, LEFT LOWER LOBE (a) PA VIEW BEFORE TREATMENT ;  
(b) LATERAL VIEW BEFORE TREATMENT ; (c) PA VIEW AFTER TREATMENT ;  
(d) LATERAL VIEW AFTER TREATMENT

It is at present difficult to state for certain the extent of healing that can take place even in chronic lesions with a considerable amount of fibrosis as a result of continuous chemotherapy. In the most common and most typical example of a chronic respiratory disease, namely, tuberculosis, continuous chemotherapy for periods of three years or more has been offered as a direct competitor to surgical resection<sup>5</sup>.

The present study was undertaken to evaluate the place of tetracycline in the management of broncho-pulmonary suppuration. Treatment was started with 250 mg. (in capsule) every 4 hr. for the first two weeks and then every six hr. until the desired result was achieved. A tablet of B-complex twice a day was given throughout the treatment. Table 1 gives a summary of the 11 cases included in the study. Before treatment was started, the predominant organisms cultured from the sputum in 9 cases were reported to be sensitive to achromycin. In three out of the four cases of lung abscess, the lesion resolved radiologically. The X-ray photographs of the cases 1 and 2 (Table 1) are given in Figs. 1 & 2. In the 7 cases of bronchiectasis, radiological improvement occurred only in the more recent and acute surrounding pneumonitis, but the basic lesion persisted.

It is noteworthy that the side effects such as nausea, vomiting and diarrhoea, often seen with chlortetracycline and oxytetracycline, were completely absent in these cases. There was never any difficulty in keeping up the treatment for as long as was necessary. Blood counts during and after the treatment did not show any toxic effects on the bone marrow.

Among the cases of bronchiectasis included in this series, three have had penicillin alone, 2 penicillin with sulphadiazine and one penicillin and streptomycin previously. The improvement, particularly in the reduction of the quantity of sputum, was not so appreciable with the earlier treatments as with achromycin therapy. Case No. 9 had a successful resection of the right middle lobe. Case No. 8 died of sensitivity to percaine during bronchoscopy.

#### ACKNOWLEDGMENT

We are indebted to Lederle Laboratories Division of American Cyanamide Co., for the supply of Achromycin, their brand of tetracycline, used in these studies.

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# Personal Experiences with Tetracycline and Carbomycin : Part III—Achromycin in Acute Amoebic Dysentery

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**The results of the use of achromycin in six cases of acute intestinal amoebiasis have been presented.**

While in the past parenteral emetine has been the mainstay in the treatment of acute amoebic dysentery, it is certainly desirable to have alternative drugs which are safer and which could be administered orally. Drugs satisfying these requirements will be particularly useful in a country where the general nutritional state is poor and most patients with amoebic dysentery show hypotension.

Except perhaps chlortetracycline which is claimed to have a direct action on the amoeba, the action of the broad spectrum antibiotics in controlling amoebic dysentery seems to be by the indirect method of inhibiting the activity of the bacteria in the intestinal lumen. Apparently, *Entamoeba histolytica* (E.H.) can assume pathogenicity only when the bacterial flora of the intestinal tract reach a certain concentration.

There are several reports<sup>1,2</sup> on the place of chlortetracycline and oxytetracycline in the treatment of acute amoebic dysentery. It is generally believed that oxytetracycline is definitely superior to chlortetracycline in the treatment of intestinal amoebiasis. Jung *et al.*<sup>3</sup> have reported on four cases treated successfully with magnamycin, one of which had shown no response previously to terramycin. It has been pointed out that "the fact that tetracycline produces fewer untoward side reactions should not be persuasive in making a decision to use it as a substitute for its two analogues without knowledge of its efficacy in the specific infection being treated. It would seem more logical to consider tetracycline as a new broad-spectrum antibiotic ; that its effectiveness in any given disease can only be demonstrated by adequate clinical trial<sup>14</sup>".

This paper gives an account of the treatment with tetracycline of six cases of acute amoebic dysentery in which the vegetative forms were demonstrated. The patients were all adults, five men and one woman.

Tetracycline, 250 mg. in capsules, was given fourth-hourly for ten days along with one tablet of vitamin B Complex with each capsule of the antibiotic. A brief summary of the cases is given below.

*Case 1*—Had the symptoms off and on for the last four months. The severity of the dysentery showed a significant improvement but patient continued to have four semi-solid stools a day. The vegetative forms disappeared on the third day of treatment. Seen four months after the conclusion of this treatment, he has been at work during this period and feeling well except for the tendency to a frequency of bowel movements.

*Case 2*—Duration of symptoms, two weeks. The abdominal pain and dysenteric stools cleared on the third day. The vegetative forms disappeared on the second day. Seen six months after discharge, he has been keeping fit without any recurrence of symptoms.

*Case 3*—A case of severe dysentery of two weeks' duration. On admission he showed considerable dehydration and had to be given intravenous saline and other supportive treatment. The vegetative forms disappeared from the stools on the second day, but the diarrhoea could not be controlled and the patient died on the fourth day of treatment.

*Case 4*—Admitted with fever, abdominal pain and dysentery of three days' duration. The vegetative forms disappeared on the second day. Motions were formed fifth and normal from the day onwards. This patient has not answered our followed-up enquiry.

*Case 5*—Had dysenteric symptoms for twenty days. From the sixth day of treatment his stools were normal and formed. No *Entamoeba histolytica* from the second day of treatment. The patient has reported that for the last six months he has had no dysentery or diarrhoea.

*Case 6*—Has had similar symptoms off and on for the last four months. From the fourth day of treatment there was no blood or mucous in the stools nor diarrhoea. No *E. histolytica* from the second day. Seven months after discharge from the hospital, she was seen in a good state of health. Motions examined now showed no *E. histolytica* or cysts.

None of the patients in the series had a systolic blood pressure above 116 mm. although their ages ranged from 27 to 53 years.

It would appear that tetracycline is as valuable as oxytetracycline in intestinal amoebiasis. One cannot be sure whether the diarrhoea noted in case No. 1 was a complication of treatment inasmuch as it persisted even after the cessation of treatment. There was no nausea or vomiting complained of by any of the patients.

Although tetracycline was used alone in this series of cases in order to judge its efficacy, it is obvious that the drug cannot be expected to have any effect on the systemic forms of the disease. It is quite essential that in any acceptable schedule of treatment for amoebiasis, the antibiotic should be combined with tissue amoebicides like chloroquine and drugs of the iodoquinoline sulphonic acid family for their action on the cysts.

## ACKNOWLEDGMENT

We are indebted to Lederle Laboratories Division of *American Cyanamide Co.*, for the supply of achromycin, their brand of tetracycline, used in this study.

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# Personal Experiences with Tetracycline and Carbomycin : Part IV—Carbomycin in Donovanosis

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**Observations on eight cases of donovanosis treated with carbomycin served to confirm earlier reports that it was an effective drug for the treatment of this disease.**

Donovanosis is a chronic, slowly progressive and mildly contagious disease of venereal origin characterized by granulomatous ulceration of the genitalia and neighbouring sites with little or no tendency to spontaneous healing. It was first recognised in India by McLeod who described the condition under the name of "Serpiginous ulcer" in Madras. The credit for discovering and describing the presence of certain intracellular bodies in the exudate from an early lesion of donovanosis in Madras belongs to Donovan, the co-discoverer of the etiological agent of kala-azar. Donovanosis should be distinguished from lymphogranuloma venereum which is a virus disease affecting the lymphatic systems of the inguino-genito-ano-rectal regions and associated with constitutional symptoms. A full description of the disease based on personal observations can be found in a recent monograph by Rajam and Rangiah<sup>1</sup>.

Whitaker *et al.*<sup>2</sup> have reported on seven patients with donovanosis treated with magnamycin. Four patients received the medication orally and three intravenously. It was apparent from the beginning that magnamycin was an effective drug for the treatment of this disease. Both routes of administration seemed equally effective. The dosage employed depended upon the size of the lesion. In five out of the seven cases, the lesions healed after a total dosage of 12 to 28 g. of the antibiotic. The patients with donovanosis expressed a feeling of well-being and satisfaction which they attributed to the drug. This preliminary study indicated that magnamycin was admirably suited to the treatment of donovanosis.

Drs. Rajam and Rangiah treated 8 cases of donovanosis with magnamycin. All the 8 patients showed donovania granulomatis in the scrapings from the typical lesions. Magnamycin was given orally at the rate of 2 tablets of 250 mg. each every six hr. for 9 to 20 days, the duration of treatment

TABLE 1—CARBOMYCIN IN DONOVANOSIS

CASE NO.	AGE	SEX	SITE	DAYS	NO. OF TABLETS	RESULTS
1.	30	F	Labia	10	80	Healed
2.	25	F	Labia, Crural fold, & Gums	10	80	Started healing
3.	25	F	Crural fold, & Perineum	10	80	Started healing
4.	42	M	Coronal sulcus, Pubis, Groins & Scrotum	10	80	Started healing
5.	15	M	Prepuce	8½	68	Started healing
6.	40	M	Prepuce, Perianal region & Crural fold	20	160	Healed
7.	36	M	Glans, Coronal Sulcus	20	80	Healed
8.	30	M	Lips, Mouth & Neck	20	160	Started healing

depending on the extent of the lesion. Table 1 gives full information regarding the distribution of the lesions, the dose and duration of therapy and the results noted in this series of cases. In case No. 6 the scrapings from the lesions were negative for *donovania granulomatis* even in 6 days of therapy. Case No. 8 had been treated previously with streptomycin without any benefit.

None of the patients showed any side reactions worthy of record. The results of treatment have been very satisfactory in all the cases. Sufficient time must, however, elapse to determine whether the beneficial effects of magnamycin would be permanent and proof against relapse. It is now more than six months after the completion of treatment. One patient has been examined again and has not had any relapse. None of the other cases had reported the recurrence of the complaint.

This study of 8 cases of donovanosis goes to confirm the claims of Whitaker *et al.*<sup>2</sup>

#### ACKNOWLEDGMENT

We are indebted to *Chas. Pfizer & Co.*, for the supply of magnamycin, their brand of carbomycin, used in these studies and to Drs. Rajam and Rangiah for their observations made on eight cases of donovanosis.

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### Discussion

DR. K. GANAPATHI: Is it a fact that carbomycin has not lived up to the expectations?

What is the comparative value of carbomycin, terramycin and fumigallin in their use for the control of amoebiasis?

DR. K. S. SANJIVI: Valuable results regarding the betterment of amoebiasis are yet to come. Of the three antibiotics oxytetracycline may supercede others in view of lesser side effects on human systems. With regard to fumigallin I have no personal experience.

COL. P. N. BHARDHAN: It is necessary to have a proper integration of variables beforehand and then successive trial of experiments with different antibiotics. The disease amoebiasis finds natural relapses and simply one year's experience with regard to an antibiotic would hardly lead to a definite conclusion.

DR. B. B. YODH: The values of broad spectrum antibiotics as amoebicides are still unknown. Relapses in cases of amoebiasis are too common to give any correct judgment.



# Oxysporin in Experimental Tuberculosis and Preliminary Observations on Toxicity and Pharmacodynamic Action of Oxysporin

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Oxysporin, an antibiotic obtained from *Fusarium oxysporum* Schlecht, strain 549, inhibits the growth of virulent tubercle bacilli *in vitro* and controls the disease process in experimental tuberculosis of mice. The antibiotic is found to be highly toxic when administered intravenously to anaesthetized dogs, but rats tolerate more than 100 mg./kg., when given intraperitoneally.

Oxysporin is a new antibiotic obtained from a strain of *Fusarium oxysporum* Schlecht, strain 549, originally obtained from the Ministry of Agriculture, Government of the Republic of Argentina<sup>1</sup>. Cultural studies on the antibiotic production from *Fusaria* in general and of oxysporin in particular have been described earlier<sup>2</sup>. The antibacterial spectrum, and the effect of this antibiotic on experimental tuberculosis in mice is outlined in this communication.

## EXPERIMENTAL AND RESULTS

### Anti-bacterial activity of oxysporin *in vitro*

The concentration inhibiting the growth of the organisms in the serial dilution technique is shown in Table 1.

All organisms were tested in nutrient broth except the shigella, streptococci and the cholera strains. The former two were tested in serum broth and the latter in peptone water.

The spectrum indicates the high susceptibility of the Gram-positive organisms to oxysporin and the slight effect of the antibiotic on cholerae and dysentery group of bacteria and no activity on the other Gram-negative micro-organisms.

### *In vitro* anti-mycobacterial activity of oxysporin

The action of oxysporin on the growth of certain saprophytic and pathogenic acid-fast bacteria has been studied. *Mycobacterium lacticola* O<sub>11</sub> and O<sub>12</sub> in nutrient broth and H<sub>37</sub>R<sub>V</sub> strain of *Mycobacterium tuberculosis* in modi-

TABLE 1—IN VITRO ACTIVITY OF OXYSPORIN

ORGANISMS TESTED	OXYSPORIN, 1 PART IN				
	1,000 parts	10,000 parts	100,000 parts	1,000,000 parts	10,000,000 parts
<i>Micrococcus pyogenes</i> var. <i>aureus</i>	—	—	—	+	+
<i>Streptococcus hemolyticus</i>	—	—	—	+	+
<i>Shigella sonnei</i>	—	—	+	..	..
<i>Shigella paradysenteriae</i>	—	+	+	..	..
<i>Salmonella typhosa</i>	+	+	+	..	..
<i>S. paratyphi-A</i>	—	+	+	..	..
<i>S. schottmuelleri</i>	+	+	+	..	..
<i>Klebsiella pneumoniae</i>	—	+	+	..	..
<i>Alcaligenes faecalis</i>	+	+	+	..	..
<i>Escherichia coli</i>	+	+	+	..	..
<i>Vibrio cholerae</i> inaba	—	+	+	..	..
<i>Vibrio cholerae</i> ogawa	+	+	+	..	..
<i>Proteus vulgaris</i> O X 19	—	+	+	..	..
<i>Proteus vulgaris</i> O X K	+	+	+	..	..
<i>Proteus vulgaris</i> O X 2	+	+	+	..	..

TABLE 2—IN VITRO ANTI-TUBERCULAR ACTIVITY OF OXYSPORIN

	OXYSPORIN, 1 PART IN				
	1,000 parts	10,000 parts	100,000 parts	1,000,000 parts	10,000,000 parts
First	—	—	—	—	—
Second	—	—	—	—	—
Third	—	—	—	—	+

—, total inhibition ; +, full growth

TABLE 3—ACTIVITY OF OXYSPORIN AGAINST *M. LACTICOLA*

STRAIN	OXYSPORIN, 1 PART IN				
	1,000 parts	10,000 parts	100,000 parts	1,000,000 parts	10,000,000 parts
O <sub>11</sub>	—	—	—	+	+
O <sub>12</sub>	—	—	—	—	+

—, total inhibition ; +, full growth

fied Youmans' medium by the surface culture method<sup>3</sup> were adopted for evaluation. The results are given in Tables 2 and 3.

Subsequent subculturing of the same inocula, after washing, on to fresh modified Youmans' medium without any antibiotic, indicated that oxysporin possesses bactericidal activity at dilutions 1: 100,000. At higher dilutions the effect was bacteriostatic.

The influence on saprophytic mycobacteria is shown in Table 3. The effect was of a lower order than that on the virulent strains.

### Stability of oxysporin

*Effect of pH*—Oxysporin has been found to be stable at a wide range of pH values, from 3.5 to 7.5, although loss in activity was observed at pH beyond 7.5. The compound was autoclaved at the various pH levels, adjusted to 7.0 before testing against *M. pyogenes*, var. *aureus* by the agar-plate method.

*Effect of body fluids and tissue extracts*—Whole blood, gastric extract and intestinal mucosa were tested for their effect on the activity of oxysporin, and the results (Table 5) indicate that the activity of the antibiotic is not appreciably affected by these agents.

### Oxysporin in experimental tuberculosis of mice

The detailed procedures of infection and the establishment of the dose-mortality curves in our strain of mice have been described previously<sup>4</sup>.

Following infection with 0.2 mg. wet weight of tubercle bacilli, the mice were separated into three groups, the second and the third were treated with dihydro-streptomycin (50 mg./kg.) subcutaneously and oxysporin (100 mg./kg.) intraperitoneally respectively, while the first group was kept as untreated control. These injections were given daily for a period of 25 days by which time all the control animals had died.

The growth response of the individual groups of mice (Fig. 1) indicates that the loss in weight observed with untreated animals is prevented and a slight increase in weight is observed in both streptomycin and oxysporin treated animals. While the untreated control group presented 100 per cent mortality, only one in oxysporin-treated had succumbed and none in streptomycin-treated group.

Immediately after the death of the animals, the chest was opened and the lungs taken out for macroscopic and microscopic study after proper fixation. The surviving animals were sacrificed on the twenty-fifth day and a similar evaluation of the lung lesions made.

Macroscopic lesions of the lungs (Fig. 2) indicate the lesser degree of involvement of the lungs and the absence of the necrotic type of lesions frequently met with in untreated group.

A comparative statement of survival time, gross amount of lesions, loss or gain in weight, percentage mortality and the nature of microscopic lesion of the experimental animals is given in Tables 6 and 7.



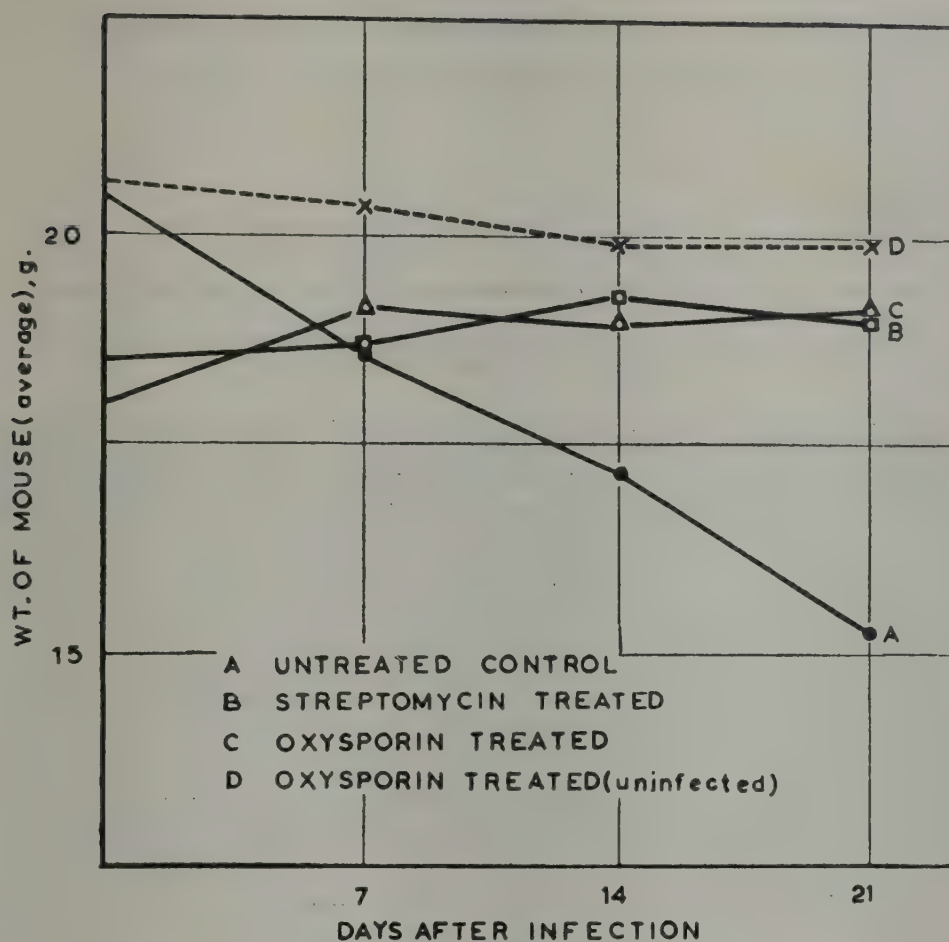


FIG. 1—EFFECT OF OXYSPORIN ON TUBERCULOSIS OF MICE

Due to the small amount of oxysporin available, larger groups of animals could not be experimented upon. Even then, from the wide disparity between the control and the oxysporin treated group as judged from growth response, mortality, degree of involvement of the lungs and nature of the microscopic lesions observed, it may be stated that oxysporin exerts a beneficial effect on the course of tuberculosis in mice.

### Toxicity of oxysporin

*Acute toxicity*—Since the drug was not soluble in water intravenous toxicity could not be tried. One hundred mg./kg. of oxysporin in olive oil given intraperitoneally caused no mortality in mice.

*Chronic toxicity*—A daily dose of 100 mg./kg. of oxysporin in olive oil was given intraperitoneally over a period of 30 days. No loss in weight, mortality or other adverse effects were noticed.

### Pharmaco-dynamic actions

*Effect on blood pressure*—This was tried in seconal sodium anaesthetized dogs, the carotid pressure being recorded on the kymograph. One, two and three mg./kg. of the drug in alcohol, given intravenously did not affect the

TABLE 4—EFFECT OF pH ON THE ACTIVITY OF OXYSPORIN

pH	3.5	4.5	5.5	6.5	7.0	7.5	8.5
Zone of inhibition of <i>M. pyogenes</i> var. <i>aureus</i> (mm.)	17	16	17	18	17	16	12

TABLE 5—EFFECT OF BODY FLUIDS AND TISSUE EXTRACTS ON THE ACTIVITY OF OXYSPORIN AGAINST *M. LACTICOLA* O<sub>11</sub>

BODY FLUID OR TISSUE EXTRACT	OXYSPORIN, 1 PART IN			
	1,000 parts	10,000 parts	100,000 parts	1,000,000 parts
Control	—	—	—	+
Blood (10%)	—	—	+	+
Gastric juice	—	—	—	+
Intestinal mucosa	—	—	—	+

—, inhibition; +, growth.

TABLE 6—ACTIVITY OF OXYSPORIN IN TUBERCULOSIS OF MICE

MICE NO.	GROUP	SURVIVAL TIME days	GROSS AMOUNT OF LESIONS IN LUNGS
1.	A-untreated control	12	Died of cross infection
2.	do.	21	3.5
3.	do.	25	3.6
4.	do.	22	3.0
5.	do.	23	3.2
6.	B-streptomycin treated	—	1.0
7.	do.	—	2.0
8.	do.	—	1.5
9.	do.	—	3.0
10.	do.	—	2.0
11.	C-oxysporin treated	25	2.0
12.	do.	—	1.0
13.	do.	—	3.0
14.	do.	—	2.0
15.	do.	—	2.5

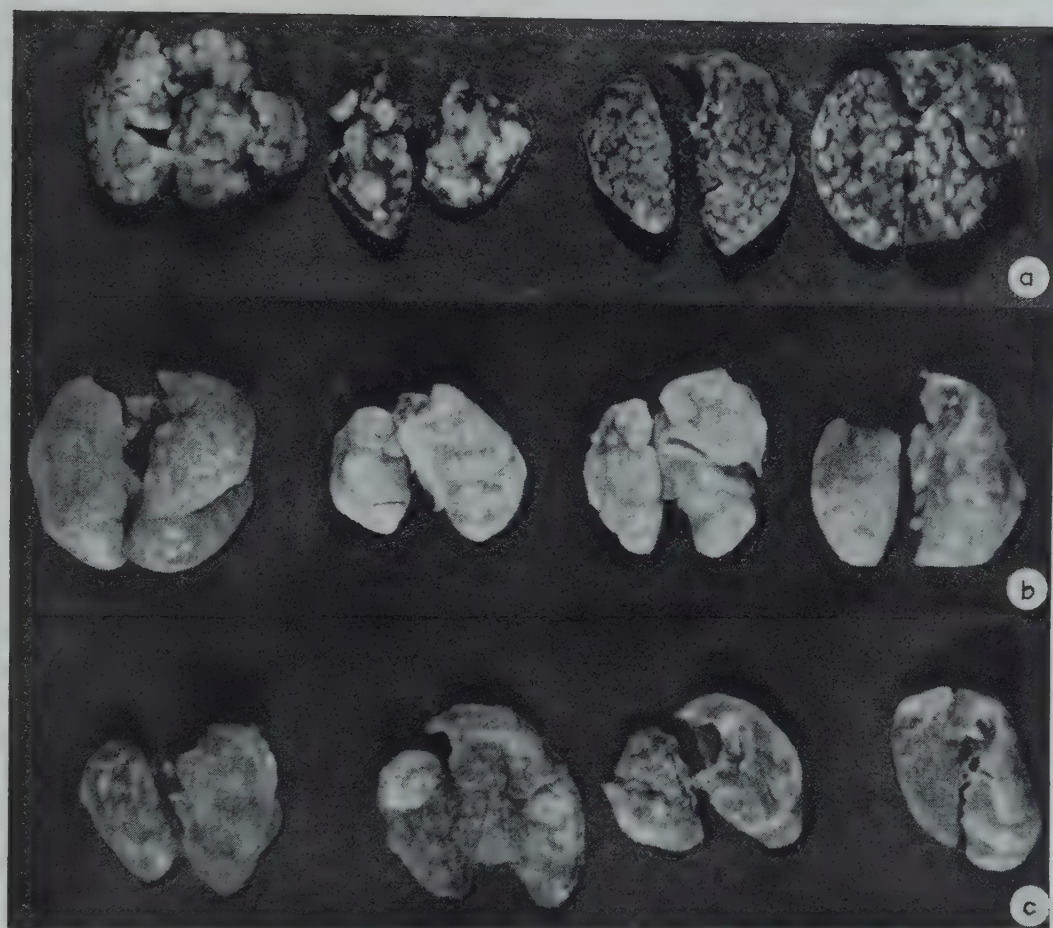


FIG. 2.—EFFECT OF OXYSPORIN ON TUBERCULOSIS OF MICE. LUNGS FROM: (a) UN-TREATED INFECTED GROUP ; (b) OXYSPORIN TREATED GROUP ; (c) STREPTOMYCIN TREATED GROUP



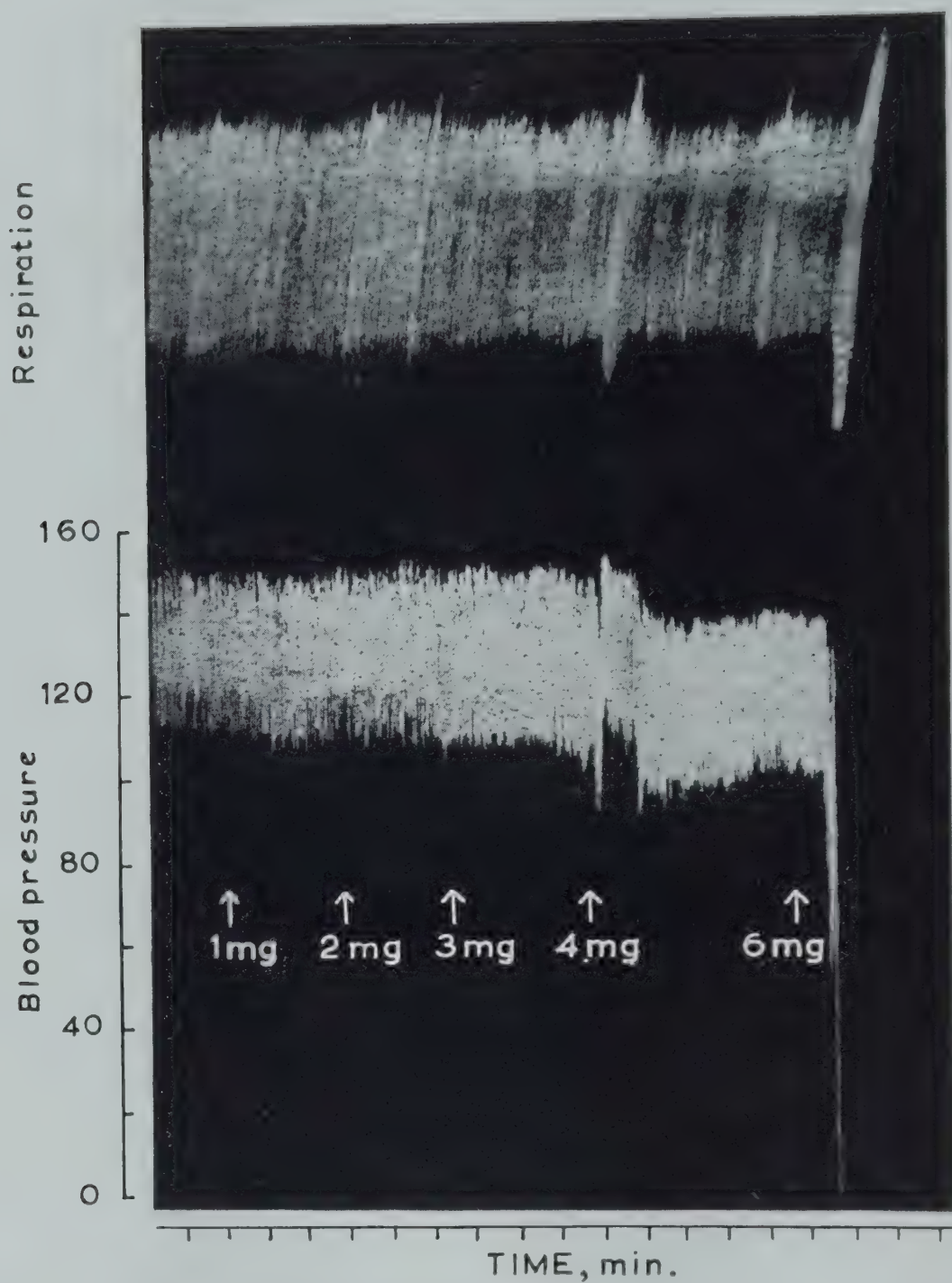
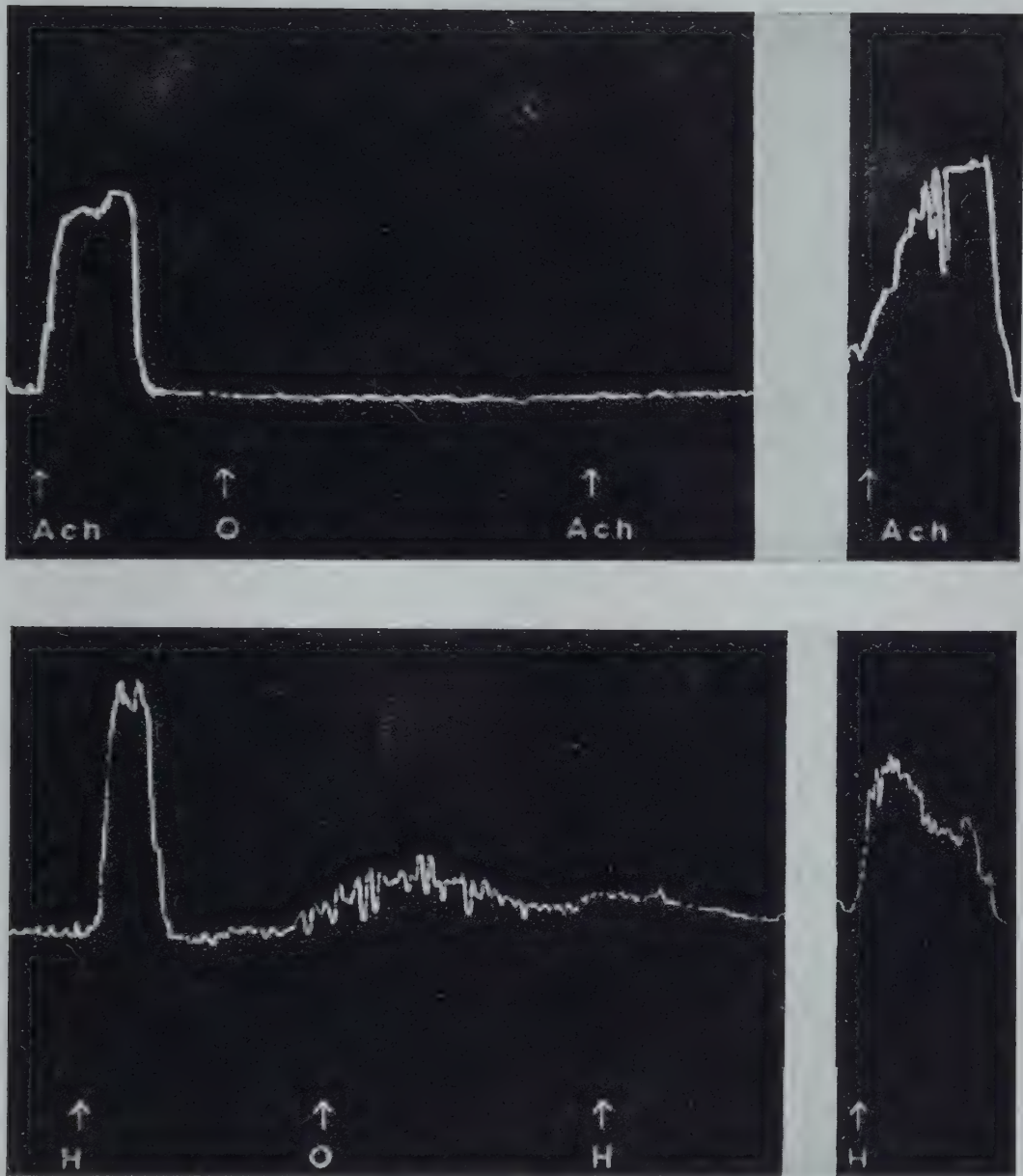


FIG. 3—BLOOD PRESSURE AND RESPIRATORY RECORDINGS (DOG WT., 6 KG., SECONAL SODIUM 30 MG./KG. 1 P ANAESTHESIA. DOSES OF OXYSPORIN GIVEN, MG./KG.)



FIGS. 4 & 5—CONTRACTION OF THE GUINEA-PIG ILEUM PREPARATION IN 15 ML. TYRODE SOLUTION (Ach, ACETYLCHOLINE CHLORIDE 0.1  $\mu$ G. INTRODUCED IN THE BATH ; H, HISTAMINE DIHYDROCHLORIDE 0.1  $\mu$ G. INTRODUCED IN THE BATH ; O, OXYSPORIN 10  $\mu$ G./ML. (FINAL CONCENTRATION IN THE BATH))

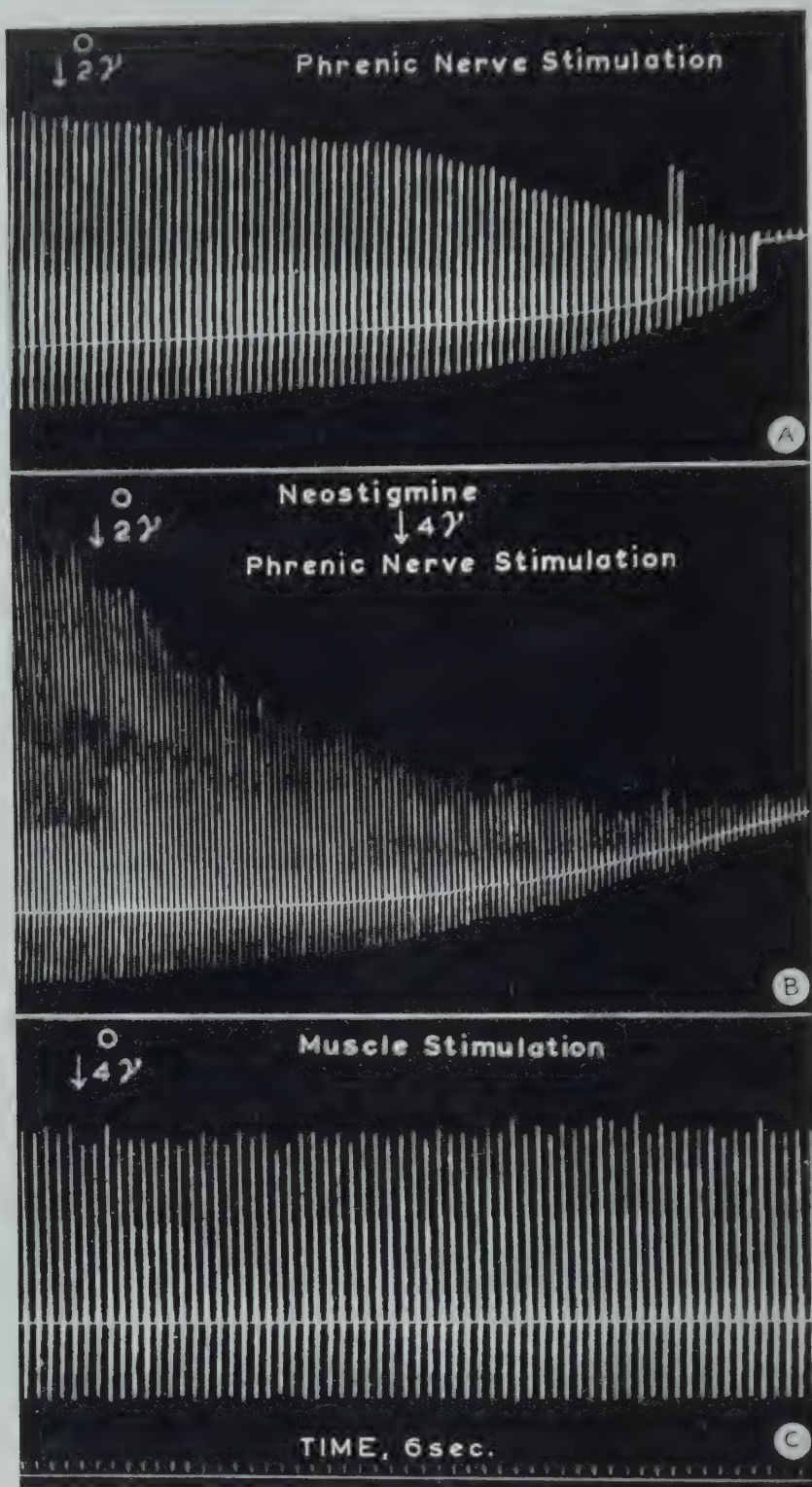


FIG. 6—EFFECT OF OXYSPORIN ON NEURO-MUSCULAR JUNCTION: RAT PHRENIC NERVE DIAPHRAGM PREPARATION [A: CONTRACTIONS DUE TO NERVE STIMULATION (O, OXYSPORIN 2 $\mu$ G.); B: CONTRACTIONS DUE TO NERVE STIMULATION AND THE EFFECT OF NEOSTIGMINE ON THE INHIBITION DUE TO OXYSPORIN (O, OXYSPORIN, 2  $\mu$ G./ML. NEOSTIGMINE, 4  $\mu$ G./ML.); C: CONTRACTIONS DUE TO DIRECT DIAPHRAGM MUSCLE STIMULATION (O, OXYSPORIN, 4  $\mu$ G./ML.)]



TABLE 7—EVALUATION OF OXYSPORIN ACTIVITY IN TUBERCULOSIS OF MICE

GROUP	NO. OF MICE	AVERAGE LOSS OR GAIN IN WT. g.	MORTALITY %	LESIONS IN LUNGS	
				Gross	Microscopic
Untreated	5	-5.2	100	3.3	EN
Streptomycin treated	5	1.4	—	2.0	P
Oxysporin treated	5	0.6	20	2.1	P

EN, exudative necrotic; P, proliferative.

blood pressure. At 4 mg./kg., an immediate transitory drop in pressure to be followed a few minutes later by a consistent drop, was noticed. With 5 mg./kg., a sudden fall in pressure ending in death resulted (Fig. 3). The same trend at similar dosages was noticed with two other dogs. Considering the fact that the effect might be cumulative in nature, experiments giving a single dose of 5 mg./kg. intravenously at the first instance was tried with the same results.

*Effects on respiration*—An increased respiratory rate and amplitude synchronizing with the blood pressure fall was observed. Death was probably due to asphyxia as a consequence of circulatory failure (Fig. 3).

### Isolated organ studies

*Guinea-pig ileum*—In dilution of 1: 100,000 in the perfusion fluid, oxysporin inhibited the contractions produced by acetylcholine and histamine (Figs. 4 and 5). A slight irritant effect resulting in increased spontaneous movements was seen in a few specimens immediately after the addition of oxysporin. The muscle recovered its original reactivity to acetylcholine and histamine, after repeated washings, only after one hour.

*Rat phrenic nerve diaphragm*—In order to understand the nature of action of oxysporin at the neuro-muscular junctions, the effect of the drug on contractions of the diaphragms induced both directly and through nerve stimulation was studied. While, in 1: 500,000 dilution in the perfusion bath, the antibiotic diminished the contractile effect of nerve stimulation, even in double this dose no diminished response to direct stimulation of the musculature was observed, indicating a possible neuro-muscular block. This diminished contractility was not antagonised by neostigmine (Fig. 6).

Oxysporin is found to be highly toxic when administered intravenously to dogs. The toxic nature of the antibiotic is also seen in its influence on rat diaphragm and the prolonged effect on the smooth musculature of the intestines. However, the antibiotic is found to manifest no visible toxic reactions even in 100 mg./kg. given intraperitoneally to rats.

## ACKNOWLEDGMENT

Grateful thanks are due to the Ministry of Agriculture of the Government of the Republic of Argentina for the generous gift of authentic strains of *Fusaria* used in these studies ; to Dr. C. V. Natarajan, Public Health Laboratories, Bangalore, for providing facilities for large-scale fermentation, to Dr. K. P. Menon for advice during the investigations and to Drs. Khambatta and J. V. Bhat for the cultures of the saprophytic mycobacteria isolated by them.

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# Protection of Chick Embryos against Vaccinia Virus by a Degradation Product of Pterygospermin

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The effect of benzylisothiocyanate—a degradation product of a plant antibiotic, pterygospermin—on the vaccinia virus grown in chick embryo has been studied. It has no action on the vaccinia virus. In concentrations of 0.2 mg. or more the drug exhibits a highly protective action on chick embryos infected with vaccinia virus as against the untreated ones.

It seems that the action of the drug is not on the virus itself but on some of its toxic components.

Though it can be confidently stated that there are effective chemotherapeutics and antibiotics against bacterial diseases, the same cannot be said of the virus diseases. Ever since their discovery, the antibiotics have been used on viruses under different conditions with little or no success. It is only one group of large viruses namely the Trachoma-Psittacosis group which is found to be susceptible to the action of broad-spectrum antibiotics like terramycin and aureomycin. In the early days of penicillin it was claimed by some that penicillin was effective against vaccinia virus. It has been observed by the author<sup>1,2</sup> that crystalline penicillin is totally ineffective against vaccinia virus. There have been claims that the terramycin-aureomycin and chloromphenicol group of antibiotics inhibit some of the viruses but definite proofs are still lacking. The beneficial effect of the antibiotics noted in some viral diseases is mostly due to their action on the superimposed bacterial infection than on the virus. But two recent antibiotics are now known to exert some action against certain viruses. They are Erlichin<sup>3</sup> derived from *Streptomyces lavandulae* exerting an inhibitory action on Influenza A & B viruses *in vitro* and the other, Netrospin<sup>4</sup> an antibiotic from *Streptomyces netropis*, exerting a protective action on mice inoculated with neurotropic vaccinia virus. The possibility of penicillin neutralising or suppressing some factors in vaccinia virus has been discussed elsewhere<sup>5</sup>.

Being interested in the subject of chemotherapy of viruses attempts were made to find out the effect of certain antibiotics on the vaccinia virus. Das, Kurup and Narasimha Rao<sup>6</sup> have shown that benzylisothiocyanate a product of decomposition of pterygospermin (the antibiotic substance present in the



root of *Moringa pterygosperma*) possesses a high antibacterial activity against a variety of organisms. A soluble compound of this substance (bisulphite addition compound of benzylisothiocyanate) was supplied by Shri Das of the Indian Institute of Science, Bangalore for these studies.

### EXPERIMENTAL

The substance in question, namely, the bisulphite addition compound of benzylisothiocyanate (BAC) was dissolved in distilled water. The stock solution contained 10 mg. per ml. from which further dilutions were made.

A local strain of Vaccinia virus found to produce 100 per cent mortality of chick embryos, 72 hr. after infection through the chorioallantoic route was used. Twelve days old fertile eggs of white leg-horn (weighing about 2 ozs.) were taken up for study. Infection of the egg was done by chorioallantoic inoculation method of Burnet and the drug was administered by the yolk sac route.

#### Tests on chick embryos

Twelve days old white leg-horn fertile eggs were selected in view of the fact that the strain of vaccinia was found to produce death of the embryos in about 3 days' time consistently. The drug was administered before, during and after infection with vaccinia virus. In addition the virus treated *in vitro* for varying periods of half an hour, one hr. and two hr. with the drug was also tried.

In the first instance, both the drug and the vaccinia virus were deposited on the chorioallantoic membrane. In higher concentrations of the drug, it was observed that the virus had not grown on the chorioallantoic membrane. But it was subsequently found that the drug had produced cloudy swelling and death of the cells of the chorioallantoic membrane and thus no substrate was available for virus growth. The liver of these treated embryos when titrated for the viral content showed the same amount of viral growth as in a control embryo indicating that the drug had no action on the virus. It was noted that all the treated embryos had died.

TABLE 1—EFFECT OF BAC ON VACCINIA INFECTED CHICK EMBRYOS

AMOUNT OF DRUG SINGLE DOSE mg.	EMBRYOS SUR- VIVED/EMBRYOS TREATED	VIRAL TITRE OF THE HARVESTED CA MEMBRANE
No drug (control)	0/6	10-5.6
1.0	6/6	10-5.8
0.5	6/6	10-5.7
0.2	3/6	10-5.6
0.1	1/6	10-5.4
0.05	0/6	10-5.5



FIG. 1—PHOTOGRAPH OF INFECTED CHICK EMBRYO UNTREATED (TOP) AND INFECTED CHICK EMBRYO TREATED WITH BENZYLISOTHIOCYANATE (BOTTOM)





In the next series of experiments, the drug in various concentrations was exhibited by the yolk sac route and the vaccinia virus infection was done by the chorioallantoic route.

The variation in viral titre has not been found to be of any statistical significance in any of the treated and control infected embryos. All the embryos which had the drug from 1.0 mg. to 0.3 mg. by yolk sac route had survived while the control infected embryos were all dead. The PD 50 was found to vary from 0.17 mg. to 0.2 mg. in a single dose and 0.1 mg. in 3 repeated doses once a day (Table 1). The dead embryos had haemorrhagic patches all over and there was even extravasation of blood in the body cavities, while it was not so in the treated embryos (Fig. 1).

This was an interesting finding as it indicated that the action of pterygospermin was not on the virus proper but probably on some of its attributes. In this connection it can be said that for a long time it was thought that any pathological change in a virus disease was due to the virus particle itself. Attention is recently being focussed on the possible existence of toxins or toxic factors in viruses. There is some evidence that there are viral toxins which are contributory factors in virus diseases.

Thompson has noted many survivals amongst neuro-vaccinia infected mice when 5 phenoxy thiouracil is exhibited to mice after infection with the neuro-vaccinia virus. In view of many substances of diverse nature producing high survival rates, it is worth while investigating whether such survivals are due to suppression of any attributes as long as the drug is exhibited or whether the drug has actually induced a genetic variation by altering the host-virus relationship. By employing a modification of Oudin's agar diffusion technique it is seen that the strain of vaccinia virus produces 4 bands in conformity with the findings of Gispén<sup>8</sup>. Pterygospermin treated strains are also being subjected to antigenic analysis to find out if there are any variations in the antigenic components.

#### ACKNOWLEDGMENT

I am greatly indebted to Dr. S. Seshagiri Rau, Director of Public Health, Bangalore, for his valuable support, encouragement and advice during the study.

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### Discussion

DR. M. J. TIIRUMALACHAR: (1) Can virus in the strict sense be called an organism as it is nucleoprotein in character?

(2) In the cases of effect of thichothecin on plant viruses, the antibiotic affects the host and brings about resistance to virus. Is it a similar case in case of pterygospermin on vaccinia virus?

DR. V. N. KRISHNAMURTHY: Virus is an organism. Host cells and viruses are interrelated and host metabolism and virus metabolism are interdependent.

DR. K. GANAPATHI: The antigenicity of the virus may be changed by the reaction of benzylisothiocyanate with the virus. The action of benzylisothiocyanate on tobacco mosaic virus is known. Since benzylisothiocyanate is a very reactive compound, is the author sure it does not affect the virus by chemical combination?

DR. P. N. NANDI: Is there any other known antibiotic which might protect chick embryo against vaccinia virus?

DR. V. N. KRISHNAMURTHY: Terramycin and other antibiotics have been tried and found to have no action on vaccinia viruses.

**Antibiotics in the Treatment of Venereal Diseases and Treponematoses.**  
R. V. RAJAM (Madras).

*J. Indian med. Ass.*, Jubilee No., 1956.—Penicillin, streptomycin, chloramphenicol, the broad-spectrum tetracycline antibiotics and carbomycin were used in the treatment of various venereal diseases. Penicillin of the repository type is the most efficacious and rapidly curative in the treatment of the two major diseases: syphilis and gonorrhoea. It is ineffective in the other three minor venereal diseases, chancroid, donovanosis and lymphogranuloma venereum.

Streptomycin is effective in gonorrhoea, chancroids and donovanosis, but on account of the tendency of the organisms to develop resistance quickly, its use should be reserved for the treatment of donovanosis only as the other broad-spectrum antibiotics, though effective are too costly for routine use.

Chloramphenicol, aureomycin, terramycin and tetracycline are equally therapeutically effective in all the five venereal diseases, but inferior to penicillin in their action on syphilis and gonorrhoea. In penicillin sensitive patients, these antibiotics may have a place and the oral route is preferred except in the case of chloramphenicol where parenteral administration is equally effective and devoid of local discomfort or pain.

Tetracycline (achromycin) appears to be the least reactive promising antibiotic in the broad-spectrum series.

Penicillin, the oldest and extensively used antibiotic seems to be the most potent sensitizer among the antibiotics. (*Abstract*)



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